THE UNIVERSITY OF ALBERTA

ALLOSTERIC INTERACTIONS ON SMOOTH MUSCLE

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1971

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Allosteric Interactions on Smooth Muscle", submitted by Allan Takao Iijima in partial fulfilment of the requirements for the degree of Master of Science.

Date: ..Nov..1./71....

ABSTRACT

It was observed that although nearly all excitatory α-receptors were apparently blocked by phenoxybenzamine, noradrenaline in the presence of methacholine, elicited a response in the guinea-pig vas deferens. The phenomenon, an apparent temporary reversal of POB block in the presence of methacholine, was not due to the complete reversal of phenoxybenzamine blockade since upon subsequent washout of the methacholine, the response to noradrenaline was still blocked. Nor could the phenomenon be attributed to an "unmasking" of alpha adrenergic receptors which had escaped blockade since the vas deferens responded equally well to phenylephrine, a relatively pure α-receptor agonist.

The final equilibrium responses to agonists, noradrenaline and methacholine, were the same irrespective of the sequential addition of the agonists. However, the equilibrium response was affected by atropine, reversible α-receptor blocking agents (phentolamine and less conspicuously by tolazine) but not by additional phenoxybenzamine.

The temporary reversal of phenoxybenzamine on α-receptors was not due to an indirect effect of methacholine since methacholine after phenoxybenzamine treatment, induced a noradrenaline response after treatment with cold storage, hexamethonium, and pretreatment of the guinea-pig with reserpine. Similarly the same reserpinized tissues, after "priming" with noradrenaline, failed to respond to tyramine.

The effect of methacholine on the α-receptor after treatment of the tissue with phenoxybenzamine was found to be a graded response. Similarly, in the presence of methacholine, the α-receptor stimulation was related to the concentration of the noradrenaline.

5-HT, which by itself gave little or no contraction, elicited in the presence of methacholine a response greater than methacholine.

It was concluded that muscarinic receptors may have an allosteric influence on the α-receptors and that the site of combination of phenoxybenzamine may not be the α-receptor itself, but a site nearby. However, further experiments involving isometric contractions and comparisons of noradrenaline responses in the presence of methacholine and potassium must be performed to confirm the hypothesis of allosteric interaction of methacholine on α-receptors.

I hold that the aim of life is to find
happiness, which means to find interest.
Education should be a preparation of life.
Our culture has not been very successful.

A. S. Neill

ACKNOWLEDGEMENTS

The author wishes to thank the following people:

Dr. R.J. Reiffenstein, my supervisor, for helping me to discover the nature of pharmacology (and the magic number '6').

Dr. D.A. Cook, for his helpful criticisms.

Cecile Triggle, for proofreading this thesis.

Dr. M. Cottle, Miss Maaike Folkinga, Mrs. Christel Krueger, Mr. Hansel de Sousa, Dr. K. Prasad, for the supply of guinea-pig vas deferens.

Mr. Fred Loeffler and Mr. Ken Burt, for the preparation of slides and photographs -- (their motto: We've only done our duty").

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I. INTRODUCTION

A. GENERAL INTRODUCTION

Theories of drug-receptor interactions using mass action laws have been proposed in order to explain dose-response relationships of agonists, antagonists, and partial agonists with the receptors. However, the problem of drug-receptor interactions is more complex and theories using mass action laws only serve as functional models, and fail to explain drug-receptor interactions at the molecular level. A case in point is that there is no *a priori* reason to believe that "competitive antagonists", which shift dose-response curves of agonists to the right and still maintain maximum response, are reacting with the same receptors as the agonists unless the agonists and antagonists are structural analogues of each other. For example, atropine, an accepted competitive antagonist of acetylcholine at the muscarinic receptor is not structurally related to this agonist. Ariens and Simonis (1967) state that:

> "In general, receptors for agonists can be competitively blocked in a functional way for an antagonist, which does not imply that the antagonist topographically blocks the receptor for the agonist. The receptor areas primarily involved in the binding of antagonists may differ from those involved in the binding of agonists."

A similar opinion was voiced by Ehrenpreis (1967) that parallel shifts in dose-response curves and apparent kinetic competitive behavior as with atropine does not entirely justify the conclusion that such drugs are acting competitively at the same receptor. Therefore, a certain class of antagonist may possess a structural feature in common with the corresponding agonist. At the receptor, a binding site for this functional group may be common to both antagonist and agonist; the remainder of the molecules, however, may occupy separate sites.

The dose-response curve obtained with most agonists is sigmoid in shape and this is considered by some researchers to represent a curve characteristic of allosteric effect and cooperative action (Watkins, 1965; Changeux et al., 1967; Karlin, 1967; Changeux and Podleski, 1968; De Robertis, 1971). However, Koshland and Neet (1968) state that sigmoid curves "are not evidence per se that cooperative subunit interactions are occurring". Waud (1968) expressed the opinion that "one cannot safely attach any fundamental significance to the shape of dose-response curves".

B. LITERATURE REVIEW

1. Enzyme Theories of Allosteric Effect

With the rapid advancement in the field of biochemistry of enzymes, two theories have been proposed, at about the same time, to explain conformational changes induced by various substrates on the enzymes.

a) Induced fit and conformation changes

Koshland (1963) advocated an "induced-fit" theory for the conformational changes at the active site of the enzyme. In his model, a substrate must be properly aligned with the catalytic sites to give reaction; any substrate which has not been so aligned will give no reaction. Inhibitors, which differ in structure from the substrate, may have affinity for the active site of the enzyme but no "intrinsic activity" since there is no proper alignment with the catalytic sites. An inhibitor molecule might be bound to the enzyme "near or far from the active site" such that the substrate is inhibited from proper alignment with the catalytic sites. However, an activator such as a hormone may overcome the inhibition by aligning the substrate with the catalytic site despite the presence of the inhibitor.

"Induced-fit" theory suggests that the presence of a ligand induces a new enzyme conformation not present in significant amounts in the absence of the ligand.

b) Allosteric effects

Monod et al. (1963) have proposed a mechanism for the regulation of protein synthesis by an "allosteric effect". In this model, regulatory agents alter the conformation of the active site on the enzyme by binding to a different, nonoverlapping site separate from the active site. The allosteric effectors, which may be cooperative (increasing the reactivity or rate of product formation) or antagonistic (decreasing the affinity of the active site for the substrate) thus regulate the formation of the enzyme-substrate complex. These reversible effectors may be structurally unrelated to the substrate and may themselves lack catalytic activity.

Allosteric effectors may be recognized and distinguished from compounds which interact with the active site by the characteristic sigmoid shape when one plots reaction velocity versus substrate concentration; under other circumstances a hyperbolic plot is obtained. It is believed that the sigmoid curve suggests multiple interacting binding sites. (see General Introduction)

2. Receptor Theories of Allosteric Effects

a) Cooperative and allosteric theories of receptors

Induction of conformational changes of receptors by agonists is not a new concept in pharmacology. Nachmansohn (1959) has postulated that acetylcholine, upon attachment to its receptor site situated on the cell membrane, induces conformational changes in

the protein molecules comprising the cell membrane. This in turn lead directly to changes in cell membrane ion permeability.

Based on the Danielli and Davson concept of the cell membrane, Watkins (1965) suggests that various excitatory drugs induce a conformational change in the structural protein of the cell membrane which in turn leads to changes in the permeability of the membrane. It was speculated that an agonist such as acetylcholine displaced polar head groups of various phospholipids such as lecithins and sphingomyelins in the membrane of the cell. This in turn leads to increase in ion permeability due to gaps left in the membrane when acetylcholine reacts with the phospholipids.

Similarly Karlin (1967) has proposed an allosteric model for the effect of acetylcholine on its receptor. When acetylcholine reacts with its receptor on the membrane, there is a change in the conformational state of the receptor which shifts the equilibrium between two states of the receptors and increases the fraction of the total receptors which have the higher affinity for the agonists. This in turn accounts for the observed slopes of the Hill plot (greater than two) which is indicative of an allosteric mechanism (see Appendix). Hill coefficient is an index of a sigmoid or cooperative effect when the slope is significantly different from one [hyberbola] (Changeux and Podleski, 1968; Nachmansohn, 1970; Whitehead, 1970; De Robertis, 1971; Rang, 1971).

Belleau (1964) has proposed a concept of conformational

changes of the receptor by drugs which he called the "macromolecular perturbation theory of drug action". In his theory of drug-induced conformational perturbation, which is applicable to both cholinergic and adrenergic systems, drugs form an addition complex with the regulatory site of the protein receptor. This transforms the receptor from a resting state to a conformationally active state and it becomes an active enzyme ("specific perturbation"). This in turn leads to the stimulus which is required for the observed biological effect. Competitive antagonists may also bind to the regulatory site to induce a conformational change which is, however, catalytically ineffective ("nonspecific perturbation"). However, partial agonists can induce conformational changes intermediate between the full agonists and antagonists.

To account for the feed-back system for noradrenaline (NA) biosynthesis, Udenfriend (1968) has suggested an allosteric effect of NA on the enzyme tyrosine hydroxylase. When nerve activity is decreased, NA accumulates in the nerve and binds to the inhibitory site situated on tyrosine hydroxylase. But upon increased nerve activity, it was hypothesized that NA is released and its binding to tyrosine hydroxylase decreases. Thus hydroxylation of tyrosine to DOPA is no longer inhibited.

Mautner (1967) supposed that receptors are flexible rather than a rigid template for the drugs and has suggested that drugs should be classified as follows:

(i) agonists: drugs which cause maximum configuration changes of the receptor.

(ii) antagonists: drugs which cannot induce a configuration change of the receptor.

(iii) partial agonists: drugs which induce a partial configuration change of the receptor.

It should be mentioned that classification of the drugs in this manner is analogous to Koshland's (1963) "induced-fit" theory for enzymes.

b) Adrenergic allosteric effects

It is generally accepted that cocaine potentiates the stimulating actions of catecholamines on smooth muscle by inhibiting catecholamine uptake into sympathetic nerve terminals and allowing greater concentration of catecholamines at the receptor sites. However, some workers have suggested that cocaine has a direct action on the receptor which may account for its supersensitivity or potentiating effect (Maxwell et al., 1966; Bevan and Verity, 1967; Greenberg and Innes, 1968; Varma and McCullough, 1969; Kalsner and Nickerson, 1969).

Nakatsu and Reiffenstein (1968) have proposed that potentiation of the adrenergic response by cocaine is due to an increased utilization of receptors resulting from an increase in "efficacy" or "intrinsic activity". Phenoxybenzamine (POB), an irreversible α-receptor blocking agent, was used to block the maximum response

of the rat vas deferens so that all unblocked α-receptors (response reduced by ~ 50%) would be activated to produce a response to a supramaximal dose of NA. It was found that cocaine increased the response to a supramaximal NA concentration. Since Vohra (1969) has shown that cocaine does not protect the α-receptors against the effects of POB, it follows that cocaine does not react directly with the α-adrenergic receptor per se in this instance, but probably reacts through an allosteric mechanism as postulated by Reiffenstein (1968) and Innes and Karr (1971).

In other studies, Varma and McCullough (1969) found that stored rat vas deferens (6-8°C for 7 days) is less sensitive to exogenous NA than fresh vas deferens. However, cocaine potentiated both fresh and stored vas deferens. Similar results were reported by Ozawa and Sugawara (1970) who found that cocaine potentiated NA response after denervation of the vas deferens.

Since cold storage (Ambache, 1946; Kosterlitz and Lees, 1964) and denervation (Birmingham, 1970) lead to degeneration of the nerves, the effect of cocaine potentiation of NA responses appear to be a direct effect rather than "blockade of uptake mechanism", since the uptake mechanism is thought to be destroyed by cold storage and denervation.

Kasuya and Goto (1968) reported that cocaine enhanced the maximum responses to NA, methacholine (MCH), K^+, and angiotensin. However, cocaine's potentiation was thought to result from a greater utilization of intracellular calcium available for muscle

contraction, rather than from a direct effect on the α-receptor.

Ergometrine by itself had no effect on the guinea-pig vas deferens but when combined with NA gave 120-200% increase in contraction over NA control values (Woodruff et al., 1969). Therefore it is conceivable that ergometrine may have caused a greater utilization of the α-receptors probably by the allosteric effect postulated by Reiffenstein (1968) for cocaine.

Tuttle and Moran (1969) found that when calcium was depleted (96%) from rabbit aorta strips by ethylene glycol bis(B-amino-ethyl ether)-N,N'-tetraacetic acid (EGTA), NA and histamine (HIST) failed to protect α-adrenergic and HIST receptors from exposure of the tissue to POB. However, the antagonists phentolamine and diphenhydramine did protect the α-adrenergic and HIST receptors from POB. In nondepleted strips, NA and HIST did protect the respective receptors against POB. Based on this evidence, Tuttle and Moran (1969) concluded that calcium is involved in the combination of the agonists with the receptor but not in the binding of antagonists. It was speculated that perhaps the antagonists do not act on the receptor per se but on sites adjacent to the receptor.

Based on experimental evidence, Moran et al. (1970) have suggested that 2-halogenoethylamines may act on a common site of calcium mobilization which is required for excitation-contraction coupling. This common site may be similar for HIST, ACH, NA and 5-hydroxytryptamine (5HT). They hypothesized that a "NA recognition site" which is linked to a "Ca^{++}-binding mobilization site" must

first be activated before Ca^{++} is available for excitation-contraction coupling. If 2-halogenothylamines react with the "Ca^{++}-binding mobilization site" rather than NA receptors ("NA recognition site"), as postulated by Moran et al. (1970), then it follows that 2-halogenoethylamines do not react directly on the receptors but possibly by an allosteric mechanism.

Using rabbit aortic strips, Kalsner (1970) reported that N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a non-equilibrium competitive antagonist of the α-receptors, gave greater blockade of the α-adrenergic response if the tissues were exposed to EEDQ together with certain amines than if EEDQ were exposed alone. Phenylephrine (PHEP), NA, and adrenaline enhanced the blockade of α-receptors whereas PHEN and short-acting haloalkylamines such as N,N-dimethyl 2-chlorophentylamine (DPA) protected against the effects of EEDQ. Kalsner concluded that EEDQ, reversible antagonist, and short-acting haloalkylamines have a common site of action and that "certain amines" increase the EEDQ-α-adrenergic receptor interaction by combining with a "sterospecific binding site which is spatially distinct from the alpha site".

c) Cholinergic allosteric effects

As far back as 1926, Clark suggested that atropine (ATR) does not block the ACH receptor per se because ATR on exposure to smooth muscle in large doses cannot be displaced with excess ACH or with repeated washings. He speculated that ATR and ACH reacted

on different receptors and that the antagonism of ATR for the muscarinic receptor is "of effects rather than combination".

As previously mentioned, drugs which appear to act competitively as noted by paralleled shifts of the dose-response curves to the right while maintaining maximum response, do not justify the conclusion that the agonist and antagonist are acting competitively at the same receptor (Ariens et al., 1964; Ariens and Simonis, 1967; Ehrenpreis, 1967).

Ariens and Simonis (1967) put forward a concept of the existence of two sites on a receptor: a "specific" or "critical site" which interacts with the pharmacodynamic grouping of the agonist, and an "unspecific" or "uncritical part" of the receptor to which the antagonists would mainly anchor. Perhaps this could explain an unusual phenomenon described by Rocha E. Silva (1969) who noted that agonists could not displace antagonists which have slow dissociation constants. However, excesses of these agonists were able to elicit maximum responses. But when the excess agonists were washed out, the preparation returned to the same level of blockade as if no excess agonist had been added. This phenomenon was labelled by Rocha E Silva (1969) as the "Charniere Theory" and he hypothesized that the temporary reversal effect of the excess agonist was due to the antagonist allowing a competitive penetration of the agonists on the "specific" or "critical site" of the receptor. This phenomenon could be illustrated with atropine-acetylcholine

and diphenhydramine-histamine systems which are classical examples of competitive antagonism. The antagonists in this system do not react at the same receptor as the agonists but rather with a portion of the receptor. Therefore competitive antagonists may be functionally competitive at the receptor but not necessarily so at the molecular level.

Similarly Ehrenpreis (1967) speculated that carbamylcholine caused an allosteric effect which allowed the antagonist (l-hysocyamine) better access to its receptor, since the antagonist was more effective when added after the agonist had been administered rather than before.

Recently another theory for the interruption of the antagonism of atropine at the muscarinic receptor has been proposed. Ellenbrock et al. (1965) and Moran and Triggle (1970) have suggested that the atropine type of antagonists to muscarinic agents interact at adjacent "accessory receptor sites" rather than directly on the muscarinic receptors even though dose-response curves show a typical competitive type of antagonism. Moran and Triggle (1970) have provided experimental evidence for the existence of adjacent accessory binding sites. They found that benzhydryl mustard (an alkylating atropine-like agent) reacted at two different sites of blockade with different recovery rates. A slowly-recovering site gave a standard shift of the dose-response curve to the right with maintenance of the maximum response and a fast recovering site caused

reduction of the maximum response. Alkylation of the slowly-recovering site (which is not directly involved in the ACH binding) induces a "conformational perturbation" of the binding site normally occupied by ACH and results in decreased affinity of ACH for its receptor. It was further suggested that atropine may also bind to this accessory site which then caused a "conformational perturbation" of the ACH binding site. These findings suggest that muscarinic receptors have subunits (allosteric sites) with stereoselectivity and hydrophobic bonding properties.

It is interesting to note that Thron and Waud (1968) found that the rate of action of atropine on the guinea-pig ileum could be accelerated by pretreatment of the tissue with dibenamine. This phenomenon was believed to arise from prevention by dibenamine of a "large fraction of the receptor pool from taking up atropine". Thus the atropine concentration is effectively increased and it thus reacts faster with the muscarinic receptor. However, another interpretation is that dibenamine may have caused allosteric effects on the "atropine receptor" allowing greater access to its receptor. Similarly, Kimura et al. (1970) reported that after POB treatment, there was increased sensitization of the calf tracheal muscle to β-adrenergic agents. There appeared to be no induced change in the "structure" of the β-adrenergic receptors, no increase in affinity of the β-agonists for the receptors, nor any relationship with the α-adrenolytic action of the β-haloalkylamines.

C. STATEMENT OF THE PROBLEM

The vas deferens of the guinea-pig is contracted by both adrenergic and cholinergic drugs and the existence of inhibitory beta receptors has been demonstrated (Large, 1965; Takagi and Takayanagi, 1965; Vohra and Reiffenstein, 1967; Ganguly et al., 1970). The peak contraction caused by high doses of adrenergic drugs such as noradrenaline (NA) is followed by a relaxation phase during which the response dips below the final equilibrium phase which in turn is lower than the peak contraction (Vohra and Reiffenstein, 1967; 1968, unpublished material; Nedergaard and Westermann, 1968) [Figure 1]. The relaxation phase is believed by Vohra and Reiffenstein (1968, unpublished material) to result from activation of inhibitory α-adrenergic receptors. The response of the vas deferens to cholinergic drugs is a rapid contraction leading to a fade as predicted by the Paton's rate theory (1961) [Figure 1].

While investigating an alternative hypothesis for the origin of the relaxation resulting from the action of noradrenaline (NA), an unusual observation was made. When all excitatory α-receptors were completely and irreversibly blocked by phenoxybenzamine (POB), treatment with methacholine (MCH) appeared to reverse, temporarily, the α-receptor blockade, allowing the usual response to NA to occur [Figure 2]. Since this appeared to be an example of allosteric alteration of α-receptors, similar to that reported by Nakatsu and Reiffenstein (1968), who presented evidence that cocaine can

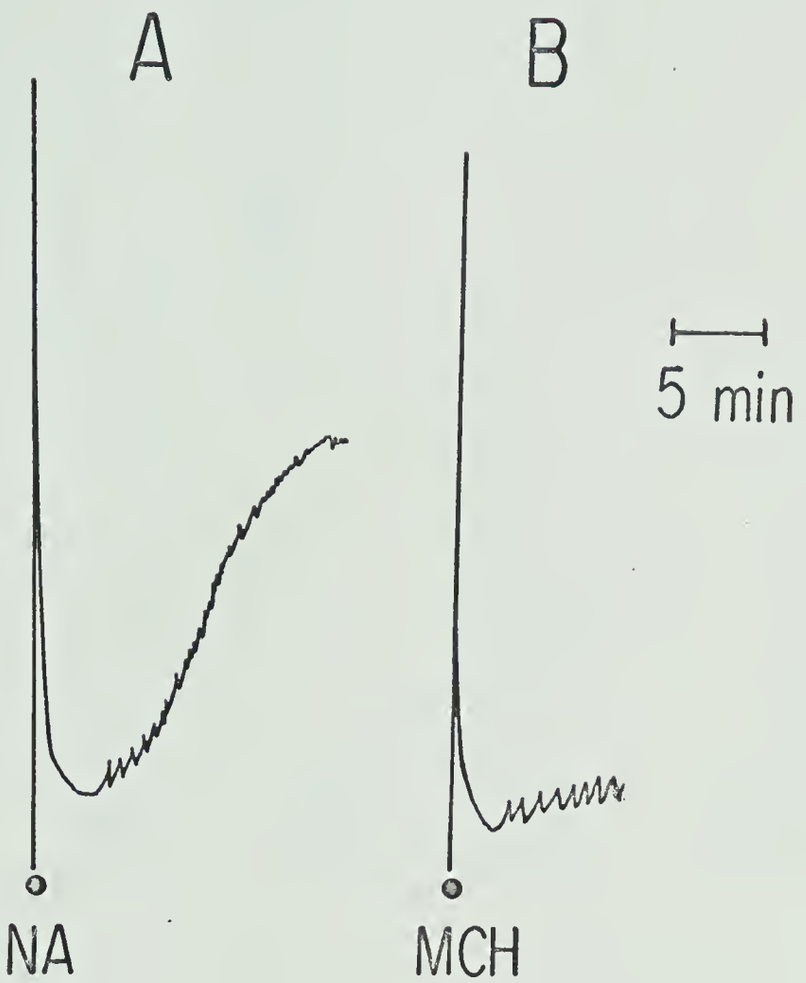

Figure 1. Typical response of the stripped guinea-pig vas deferens to adrenergic (A) and cholinergic (B) drugs.

The response to NA (A) shows a rapid peak followed by a relaxation phase which dips below the final equilibrium which in turn is lower than the peak. The response to MCH shows a rapid peak followed by a fade (B).

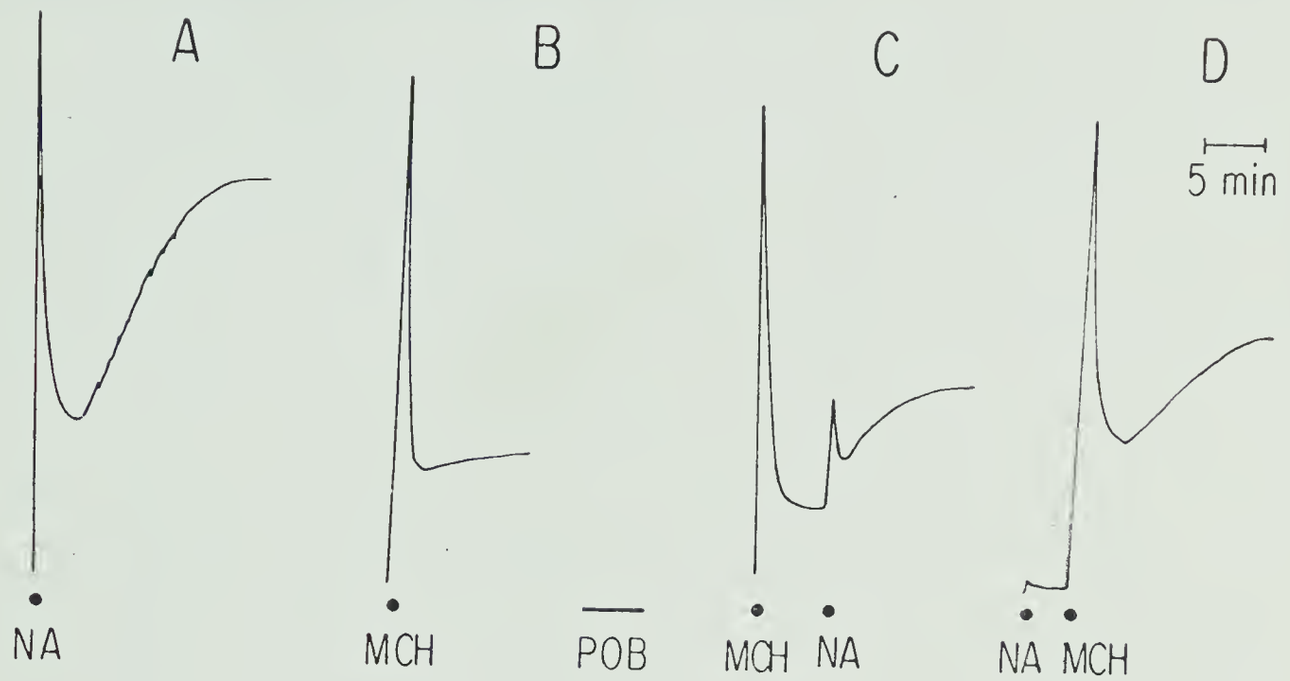

Figure 2. The temporary reversal of α-receptor blockade by phenoxybenzamine in the presence of methacholine.

A and B represent typical responses of the guinea-pig vas deferens to supramaximal doses (sup) of noradrenaline and methacholine (MCH) respectively. After exposing the tissue to phenoxybenzamine, NA in the presence of MCH appears to temporarily reverse the effect of POB (C). Upon subsequent washout, the response to NA was still blocked (D). Note that NA-plus-MCH elicited a typical NA response (A). Breaks in the diagram represent washings.

allosterically affect the receptor (see also Reiffenstein, 1968 and Innes and Karr, 1971), the pharmacological relationships of this POB-reversal effect of MCH were investigated in an attempt to determine the nature and origin of this effect.

II. METHODS AND MATERIALS

A. MATERIALS

1. Apparatus

The apparatus used in this experiment is illustrated in Figure 3. The 10 ml muscle bath in which the vas deferens is suspended was surrounded by a water jacket maintained at $34^{o}C \pm 2$ by a heater that was thermostatically controlled. The solution in the muscle bath was changed by releasing a pinchcock and fresh Krebs solution (which was kept in a separate thermostatically controlled water bath) was introduced from the top of the organ bath with a pipette. The organ bath and the stock of Krebs solution were aerated with 95% O_2 and 5% CO_2 during the entire experiment. The organ bath aeration was via the bottom of the hollow "holding rod" which also anchored the tissue in the bath.

2. Krebs solution

Krebs solution, which was prepared daily from deionized and glass-distilled water, had the following composition: Na^{+}, 138.5 mM; K^{+}, 4.36 mM; Ca^{++}, 2.47 mM; Cl^{-}, 127.4 mM; HCO_3^{-}, 21.9 mM; H_2PO_4, 1.16 mM and glucose, 49.2 mM.

Since the vas deferens was to be exposed to the sympathomimetic drugs for more than 20 minutes, 4.7×10^{-5} M disodium ethylenediamine tetraacetate (EDTA) was added to the Krebs solution

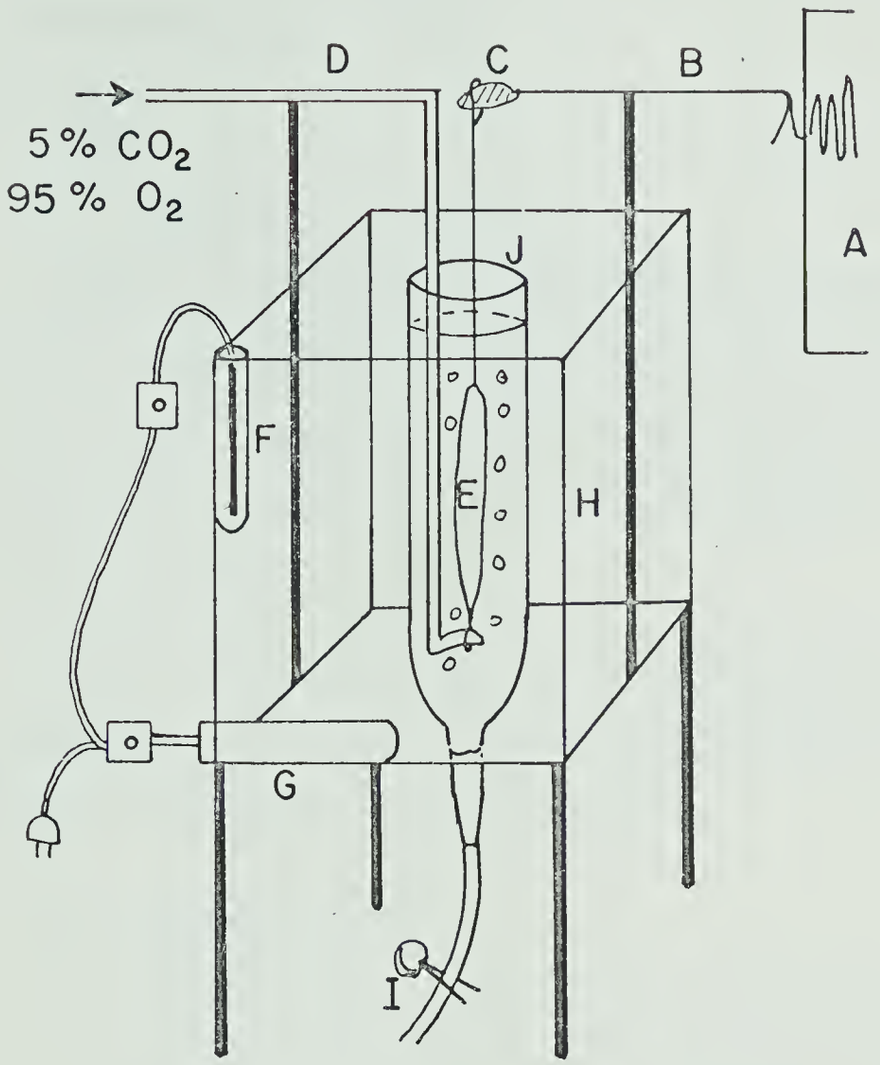

Figure 3. Apparatus

A: kymograph
B: frontal writing level
C: 0.5 gm counter-weight
D: hollow holding rod
E: vas deferens
F: thermostat
G: heater
H: water bath
I: pinchcock
J: organ bath

to decrease spontaneous oxidation of the adrenergic drugs (Trendelenburg, 1965; Nedergaard et al., 1968).

3. Drugs

a) Preparation of drugs

Drugs were prepared by dissolving in saline solution and were kept in an ice bath to prevent deterioration until used. Similarly, other precautions were taken to prevent degradation of the drugs as outlined by the staff of the Department of Pharmacology, University of Edinburgh (1968).

Drugs that remained after the experiments were stored in the refrigerator and used for succeeding experiments but were discarded if stored for more than 3 days.

b) Sources of drugs

1. Phenoxybenzamine hydrochloride (POB) stock solution was made by dissolving in 4 ml ethanol, 4 ml ethylene-glycol, adjusted to pH 4 with 2N HCl, and q.s. to 20 ml with saline. - Smith, Kline and French
2. Noradrenaline bitartrate (NA) - Winthrop Laboratories
3. Tolazoline hydrochloride (TOL) - Mt. Royal Chemicals Ltd.
4. Methacholine chloride (MCH) - Merck and Company Inc.
5. Phentolamine hydrochloride (PHEN) - Ciba Company Ltd.
6. Hexamethonium chloride (C_6) - May and Baker, Ltd.
7. Atropine sulfate (ATR) - Matheson, Coleman and Bell

8. Disodium ethylenediamine tetraacetate (EDTA) - Fisher Scientific Co.
9. Reserpine (RES) - Ciba Company Ltd.
10. 5-Hydroxytryptamine creatinine sulfate (5-HT) - Winthrop Laboratories
11. Phenylephrine hydrochloride (PHEP) - Winthrop Laboratories

All drugs were expressed in terms of Molarity (M) of their salts except for reserpine which was expressed in mg/kg.

B. METHODS

1. Preparation of the Vas Deferens

Male guinea-pigs weighing between 250-750 grams were killed by cervical dislocation. An incision was made in the abdomen and the intestines were drawn out of the abdomen and removed to one side. The vas deferens was quickly removed from the cavity and dissected free from mesenteric investment and placed in a shallow Petri dish containing Krebs solution aerated with 95% O_2 and 5% CO_2. The vas deferens was then stripped of the serous coat to increase the sensitivity to all drugs by allowing greater access of the drugs to the smooth muscle cells (Bentley and Sabine, 1963; Thoa and Maegwyn-Davies, 1968; Nedergaard and Westermann, 1968) with the aid of a 4-power illuminating magnifier. In removing the serous coat, it is now likely that all ganglion cells were removed (Ferry, 1967; Merrillees et al., 1963; Graham et al., 1968; Birmingham, 1970).

The vas deferens was then mounted in the 10 ml organ bath with the epididymal end connected to a writing arm and the prostatic end to the hollow holding rod. The vas deferens was then allowed to relax and equilibrate for 1/2 hour or until the baseline had stabilized. When no drugs were being added to the organ bath, the bath solution was replaced with fresh Krebs solution every 15 minutes.

Isotonic contractions under 0.5 gram tension were magnified about 3 times by an isotonic frontal writing level and recorded on a smoked kymograph paper moving at the rate of 2.0 mm/min.

2. Statistics

Student's t-test for paired data was used for test of significance of difference using an Olivetti programma 101 (program prepared by Reiffenstein, unpublished). Values for 't' were obtained from statistical tables from Ferguson (1966). Mean and standard error were similarly obtained using the Olivetti programma 101.

3. Interpretation of Tables and Graphs

Unless stated otherwise, all tables represent equilibrium responses (mean $\pm$ standard error) which were measured in mm from the baseline and are represented by vertical arrows in the figures. All figures are traced drawings typical of the response under experimental conditions and breaks in the diagram represent washings.

III. RESULTS AND DISCUSSION

A. EFFECT OF SEQUENCE ON THE ADDITION OF THE AGONISTS ON THE FINAL EQUILIBRIUM

Since Ehrenpreis (1967) had found that antagonist was more effective after the agonist had been added rather than before, experiments were performed in order to determine whether there was any significant difference in contraction when the sequence of addition of the agonists, NA and MCH, was varied.

After treatment of the tissue with POB ($4.4 - 11.8 \times 10^{-8}$M) for 5 minutes, the final equilibria contractions when NA was added before MCH were compared to those when NA was added after MCH. Similarly, comparisons were made between the final equilibrium response to MCH and NA in the presence of MCH. Measurement from the baseline to the final equilibrium response showed no significant difference ($p > 0.20$) irrespective of the sequence of the addition of the agonists (Figure 4, Table I) i.e., whether the NA was added after the MCH-contractions had reached equilibrium or prior to MCH. However, the comparison of the final equilibrium response due to MCH alone, and that due to NA in the presence of MCH showed that the final equilibrium response due to the latter was significantly greater than MCH alone ($p < 0.001$).

It seems reasonable to suppose that if MCH is affecting the α-receptors after treatment of the vas deferens with POB, then the

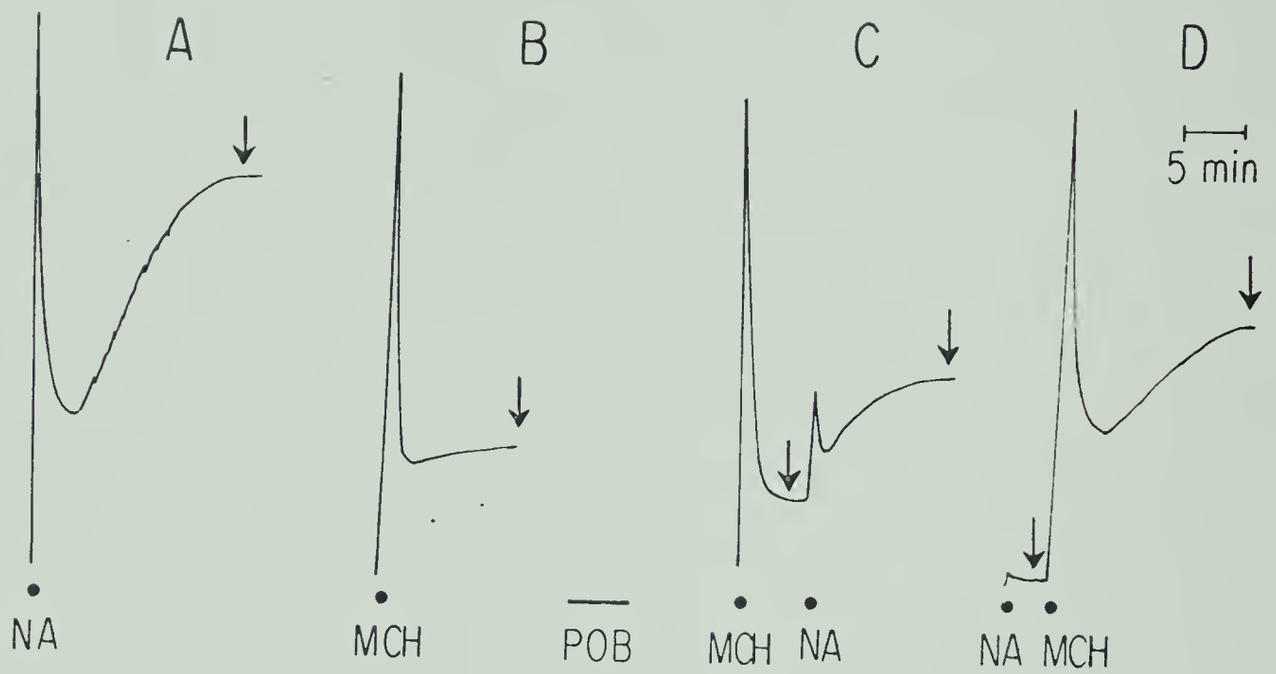

Figure 4. The effect of sequence on the addition of the agonists, noradrenaline (NA) and methacholine (MCH), on the final equilibrium response after treatment with phenoxybenzamine.

A and B represent typical responses of the stripped vas deferens to supramaximal doses (sup) of NA (14.8 - 17.8 x 10^{-5}M) and MCH (5.1 - 25.5 x 10^{-5}M) respectively. After exposure of the tissue to POB (4.4 - 11.8 x 10^{-8}M) for 5 minutes and subsequent washouts, NA (sup) in the presence of MCH (sup) elicited a response (C). Upon subsequent washouts, the response to NA (sup) was still blocked (D) and it was irrelevant whether the NA (sup) was added before or after MCH (sup), since the final equilibrium was the same. Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE I

The effect of sequence of agonists (NA + MCH) on final equilibria responses after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline					
Control[a]		After Treatment with POB			
1 NA	2 MCH	3 (a) MCH	+ (b) NA	4 (a) NA	+ (b) MCH
43.00 ± 4.10	20.27 ± 2.30	10.53[b] ± 1.77	26.73[b,c] ± 3.01	0.60 ± 0.16	25.07[c] ± 2.97

† Mean ± standard error from 8 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3(a): equilibria responses to MCH

3(b): equilibria responses to MCH-plus-NA

4(a): equilibria responses to NA

4(b): equilibria responses to NA-plus-MCH

[b] Significant difference ($p < 0.001$)

[c] Not significant ($p > 0.20$)

final equilibrium response should be the same irrespective of whether MCH is added before or after NA.

Since cholinergic and adrenergic systems are generally accepted as being anatomically and pharmacologically distinct, then any contraction recorded in the presence of a cholinergic agonist should be quite independent of that induced by an adrenergic agonist and vice-versa. If this were the case, then the NA response after treatment of the vas deferens with POB should be the same irrespective of the addition of MCH. But this is not the case. Since the equilibrium response after MCH-plus-NA is greater than after MCH alone, then there is a possibility that MCH may be inducing an effect on the α-receptor since all α-receptors were previously blocked by POB.

The observed "phenomenon" of the apparent reversal of POB block in the presence of MCH could not be due to the "reversible phase" of POB blockade (Moran et al., 1969) since upon subsequent washout of the MCH, the response of NA was still blocked. The "phenomenon" could not be attributed to the existence of "spare" α-receptors since no reports have been published for their existence in the guinea-pig vas deferens. Nor could the "phenomenon" be attributed to an "unmasking" of alpha adrenergic receptors in the tissue which had escaped blockade by the stimulation of the beta receptor by NA (Garrett et al., 1966; Yamamura and Horita, 1968, 1969; Nickerson, 1970), since the tissue responded also to supra-maximal doses of phenylephrine (PHEP) (Figure 5, Table II), a

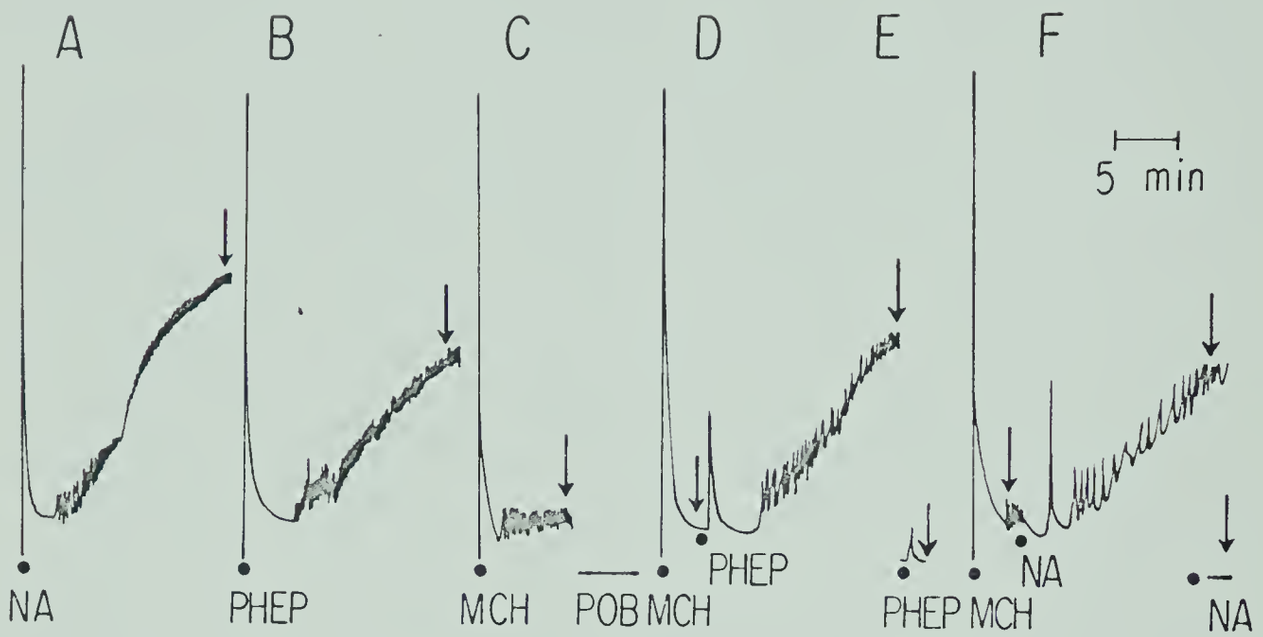

Figure 5. Comparison of noradrenaline and phenylephrine (PHEP) equilibria responses in the presence of methacholine.

A, B and C represent typical responses of the stripped vas deferens to supramaximal doses (sup) of NA (14.8 - 10^{-5}M), PHEP (4.9 x 10^{-5}M) and MCH (5.1 x 10^{-5}M) respectively. After exposure of the tissue to POB (8.8 x 10^{-8}M) for 5 minutes and subsequent washouts, PHEP (sup) in the presence of MCH (sup), elicited a response (D). However, on subsequent washouts, the response to PHEP (sup) was minute (E). Similarly NA (sup) in the presence (sup) elicited a response (F) but by itself failed to contract the tissue. Therefore, the temporary reversal of α-receptors blocked by POB does not appear to be attributed to an "unmasking" of α-receptors which had escaped blockade by POB. Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE II

Comparison of noradrenaline and phenylephrine with methacholine after treatment with phenoxybenzamine

Measurement of Equilibrium (mm)† from the Baseline								
Control[a]			After treatment with POB					
1 NA	2 PHEP	3 MCH	4 (a) MCH	4 (b) MCH + PHEP	5 PHEP	6 (a) MCH	6 (b) MCH + NA	7 NA
55.17 ± 4.65	49.67 ± 7.07	23.00 ± 6.42	23.33[b] ± 5.14	46.00[b,c] ± 5.29	1.00 ± 0.00	22.00 ± 4.99	43.33[c] ± 0.22	0.50 ± 0.22

† Mean ± standard error from 3 animals

[a] Equilibria responses to supramaximal doses of NA (noradrenaline), PHEP (phenylephrine) and MCH (methacholine)

Column 4(a): equilibria responses to MCH

4(b): equilibria responses to MCH-plus-PHEP

5 : equilibria responses to PHEP

6(a): equilibria responses to MCH

6(b): equilibria responses to MCH + NA

7 : equilibria responses to NA

[b] Significant difference ($p < 0.001$)

[c] No significant difference ($p < 0.20$)

relatively pure α-receptor agonist (Innes and Nickerson, 1970).

Comparison of the final equilibrium of MCH and MCH-plus-PHEP showed a significant increase ($p < 0.001$) in the latter, but there was no significant difference ($p < 0.20$) when final equilibrium of METH-plus-NA and METH-plus-PHEP were compared.

B. EFFECT OF TOLAZOLINE (TOL) AND PHENTOLAMINE (PHEN) ON MCH-INDUCED REVERSAL OF POB BLOCKADE OF α-RECEPTORS

TOL and PHEN, both reversible α-receptor blocking agents, were added during the equilibrium phase of the response to NA in the presence of MCH, to determine whether the NA was reacting with the α-receptors. If the equilibrium response of NA in the presence of MCH is affected by these blockers (TOL and PHEN), the following possibilities may provide an explanation:

1) there are α-receptors which are resistant to POB but not to reversible alpha-receptor blocking agents.
2) α-receptors blocked by POB have been unblocked by MCH, but the effects of the reversible blockers are not altered by MCH.
3) MCH uncovers new α-receptors (not blocked by POB when "uncovered") which are then blocked by reversible antagonists.

If, however, the response to NA is not affected by the reversible α-receptor blockers, then there is a possibility that there are α-receptors which are resistant to known α-receptors blocking agents (reversible and irreversible) or that MCH uncovers new α-receptors which are resistant to all types of α-blocking agents or alternately NA is then not acting on "α"-receptors at all.

The tissue was blocked with POB (5.9 - 20.3 x 10^{-8}M for 5 minutes) as previously described (Section A). As a control, TOL

and PHEN were added during the equilibrium phase of the response to NA supramaximal doses without prior treatment with POB. As in Section A, the equilibrium phase of the contractions to the agonists was compared before and after the addition of the reversible α-receptor blocking agents. A significant blocking effect of PHEN (7.9×10^{-5}M, $p < 0.001$) (see Figure 6, Table III) and TOL ($5.1 - 12.7 \times 10^{-4}$M, $p < 0.02$) (Figure 6, Table IV) was observed in 6 POB treated preparations. However, in 5 of these 12 vas deferentia (a pair from 6 animals) TOL was shown to have very little effect.

In control animals (n=4), TOL ($12.7 - 19.1 \times 10^{-4}$M) did not affect the final equilibrium response ($p < 0.10$) but in 8 control experiments PHEN ($3.2 - 6.3 \times 10^{-5}$) reduced the response of the vas deferens to NA ($p < 0.001$) (see Figure 7, Table V). The response to TOL in all cases showed a drop in NA equilibrium response but in most cases this was followed by a slow return to the original contraction level. However, the addition of PHEN always resulted in sustained decrease in equilibrium response (Figure 6 and 7).

PHEN and TOL reduced the equilibrium phase of the NA response in the presence of MCH even though nearly all the α-receptors had been blocked by POB. It is therefore possible that α-receptors which were not blocked by POB or were resistant to POB are "new" or reactivated receptors, which were uncovered by MCH. Distinction

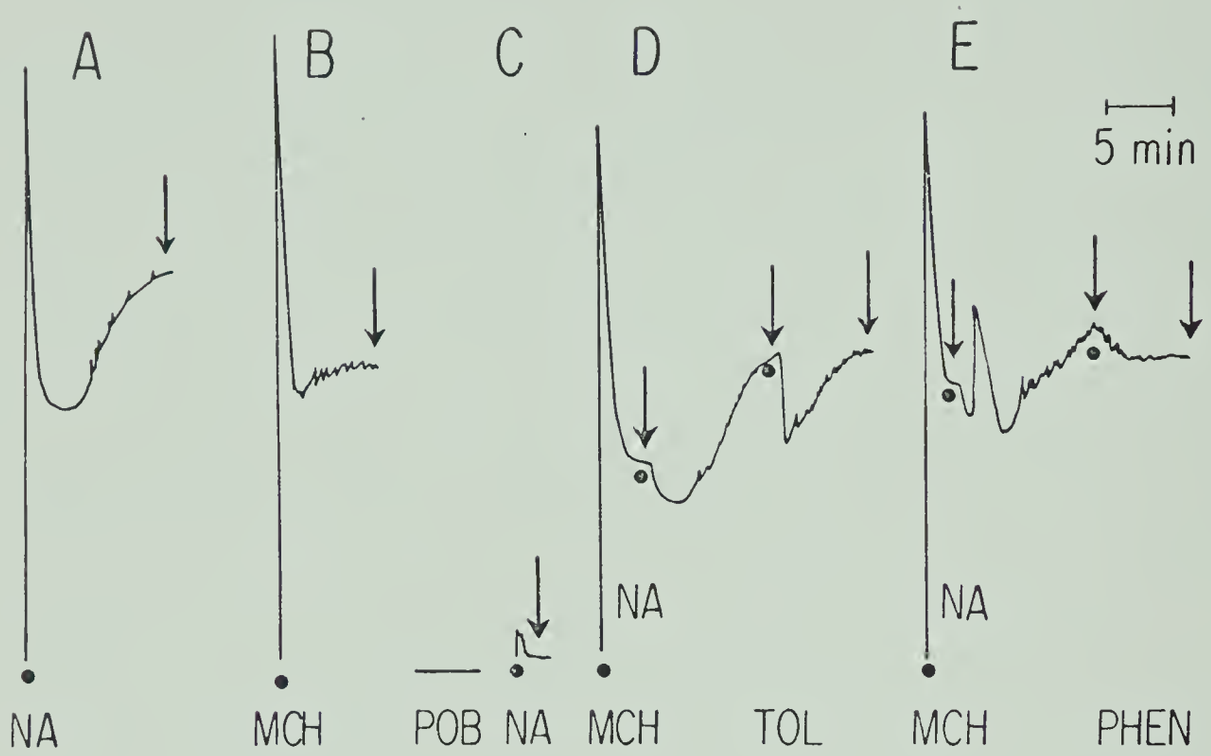

Figure 6. The effect of tolazoline (TOL) and phentolamine (PHEN) on methacholine-induced reversal of phenoxybenzamine blockade of α-receptors.

A and B represent typical responses of the stripped vas deferens to supramaximal doses (sup) of noradrenaline (14.8 - 297.0 x 10^{-5}M) and methacholine (5.1 - 25.5 x 10^{-5}M) respectively. After exposure of the tissue to POB (8.8 - 20.3 x 10^{-8}M) for 5 minutes, the response to NA (sup) was almost completely blocked (C). NA (sup) in the presence of MCH (sup) elicited a response which was subsequently blocked by TOL (5.09 - 12.7 x 10^{-4}M). However, it was followed by a slow return to the original contraction level (D). The addition of PHEN (3.2 - 7.9 x 10^{-5}M) to MCH-plus-NA equilibrium resulted in a sustained decrease in equilibrium response (E). Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE III

The effect of phentolamine (PHEN) on methacholine-plus-noradrenaline equilibria responses after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline			
Control[a]		After treatment with POB	
1 NA	2 MCH	3 (a) MCH + NA + (b) PHEN	4 NA
51.83 ± 4.88	31.08 ± 5.62	31.25[b] ± 5.11	23.50[b] ± 4.23	0.92 ± 0.19

† Mean ± standard error from 6 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3(a): equilibrium response to MCH-plus-NA

3(b): equilibrium response to MCH-plus-NA-plus-PHEN

4: equilibrium response to MCH-plus-NA

[b] Significant difference ($p < 0.001$)

TABLE IV

The effect of tolazoline (TOL) on methacholine-plus-noradrenaline equilibria responses after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline			
Control[a]		After treatment with POB	
1 NA	2 MCH	3 MCH (a) + NA + (b) TOL	4 NA
45.16 ± 3.92	22.50 ± 5.14	31.17[b] ± 4.21	28.92[b] ± 4.32	0.75 ± 0.18

† Mean ± standard error from 6 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3(a): equilibrium response to MCH-plus-NA

3(b): equilibrium response to MCH-plus-NA-plus-TOL

4: equilibrium response to NA

[b] Significant difference ($p < 0.02$)

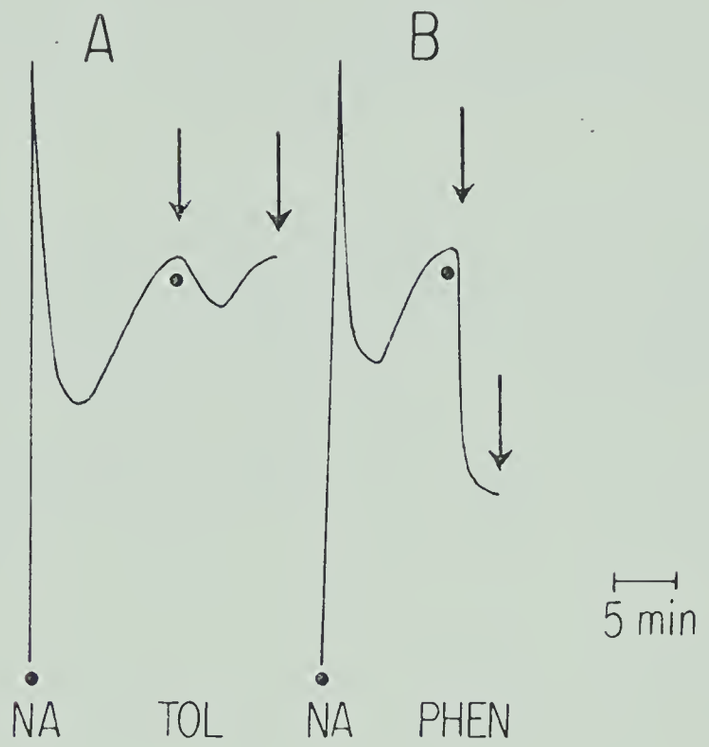

Figure 7. The effect of tolazoline (TOL) and phentolamine (PHEN) on noradrenaline equilibria responses.

The addition of TOL (12.7 - 19.1 x 10^{-4}M) to the equilibrium response to supramaximal doses of NA (17.8 - 297.0 x 10^{-5}M) decreased the equilibrium. However this was followed by a slow return to the original contraction level (A). The addition of PHEN (7.9 - 63.0 x 10^{-6}M) to supramaximal doses of NA (14.8 - 297.0 x 10^{-5}M) resulted in a sustained decrease in equilibrium response (B). Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE V

The effect of tolazoline (TOL) and phentolamine (PHEN) on noradrenaline equilibria responses

Measurement of Equilibrium (mm)† from the Baseline			
NA + TOL		NA + PHEN	
42.40[a]	35.00[a]	40.91[b]	25.93[b]
±	±	±	±
6.46	7.62	3.79	2.89

† Mean ± standard error from 4 animal (left column) and 8 animals (right column)

Left column: equilibria responses to supramaximal doses of noradrenaline (NA) and its change upon subsequent addition of TOL

[a] No significant difference ($p < 0.10$)

Right column: equilibria responses to supramaximal doses of noradrenaline (NA) and its change upon subsequent addition of PHEN

[b] Significant difference ($p < 0.001$)

between these possibilities cannot be achieved on the basis of present experimental evidence.

The inability of TOL to maintain a decrease in equilibrium contraction and its return to the initial equilibrium response may result from a stimulation of the histaminic and/or cholinergic receptors (Nickerson, 1970). Therefore, the use of TOL as a α-receptor blocking agent in the guinea-pig vas deferens is unsatisfactory.

C. EFFECT OF ADDITIONAL POB AT NA-PLUS-MCH EQUILIBRIUM

Further experiments were carried out to determine if α-receptors which had been "unblocked" by MCH could be blocked by additional phenoxybenzamine (POB_2). Unless the MCH present prevented the POB_2 from combining with and/or blocking the α-receptors, then POB_2 should slowly reduce the equilibrium response despite the "protecting" effect of NA.

POB_2 ($5.7 - 8.8 \times 10^{-8}$M) was added during the equilibrium phase of the response to MCH-plus NA tissues previously treated with phenoxybenzamine (POB_1) ($8.8 - 17.7 \times 10^{-8}$M). The final equilibrium contractions (height of contraction from the baseline) due to NA-plus-MCH equilibrium response (after the initial POB_1) did not reduce this response (Figure 8, Table VI) when tested in 4 paired preparations. Upon subsequent washout, comparison of the final equilibrium response of MCH-plus-NA after the initial dose of POB_1 and the final equilibrium response after the additional dose of POB_2 resulted in significant decrease in equilibrium response ($p < 0.05$). However, the fact that the response to NA is still marked in the presence of MCH when compared to MCH alone ($p < 0.001$) after the addition of the second dose of POB_2 might imply that the α-receptors uncovered by MCH are relatively resistant to the action of POB_2 (at least in the presence of MCH and NA). Comparison of MCH contraction after both doses of POB showed that there was a significant decrease in MCH response ($p < 0.01$). The addition of

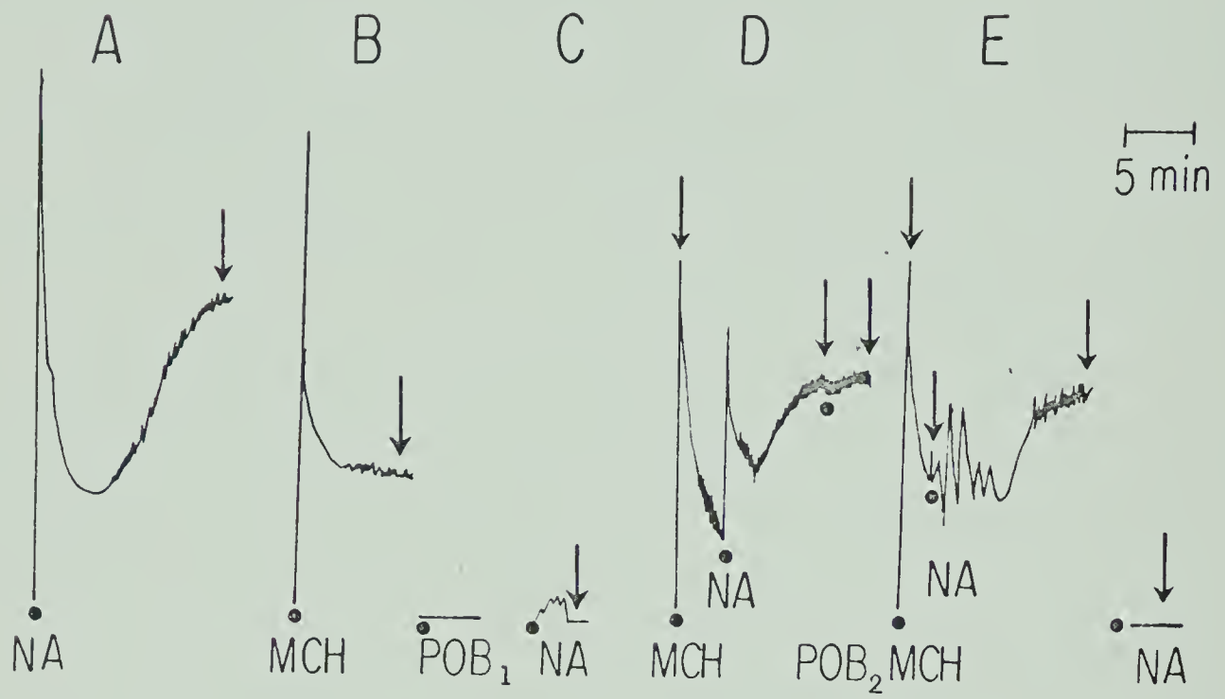

Figure 8. The effect of additional phenoxybenzamine (POB_2) at methacholine-plus-noradrenaline equilibrium after an initial exposure to phenoxybenzamine (POB_1).

A and B represent typical responses of the stripped vas deferens to supramaximal doses (sup) of NA (14.8 - 297.0 x 10^{-5}M) and methacholine (5.1 - 15.3 x 10^{-5}M) respectively. After exposure of the tissue to POB_1 (8.8 - 14.7 x 10^{-8}M) for 5 minutes and subsequent washouts, the response to NA (sup) was minute (C). However, in the presence of MCH (sup), NA (sup) elicited a response which was not affected by additional POB_2 (8.8 x 10^{-8}M) at equilibrium (D). Upon subsequent washouts, NA (sup) in the presence of MCH (sup) still elicited a response (E) even though the response to NA (sup) alone was essentially zero. Therefore, the α-receptors uncovered by MCH are apparently resistent to POB. Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE VI

The effect of additional phenoxybenzamine (POB_2) at methacholine-plus-noradrenaline equilibrium after an initial treatment with phenoxybenzamine (POB_1)

Measurement of Equilibrium (mm)† from the Baseline									
Control[a]		After treatment with POB_1							
1 NA	2 MCH	3 NA	4 (a) MCH	+ (b) NA	+ (c) POB_2	5 (a) MCH	(b) +	(c) NA	6 NA
58.12	36.50	0.62	69.38[b]	35.61	35.6	54.75[b]	12.88[c]	25.25[c]	0.50
±	±	±	±	±	±	±	±	±	±
6.04	8.51	0.26	4.76	3.39	3.34	3.94	3.46	5.32	0.19

† Mean ± standard error from 4 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3: equilibrium response to NA

4(a): initial contraction to MCH

4(b): equilibrium response to MCH-plus-NA

4(c): equilibrium response after the addition of POB_2 to MCH-plus-NA equilibrium

5(a): initial contraction to MCH after POB_2

5(b): equilibrium response to MCH

5(c): equilibrium response to MCH-plus-NA'

6: equilibrium response to NA after POB_2

[b] Significant difference ($p < 0.01$)

[c] Significant difference ($p < 0.001$)

POB_2 during NA-plus-MCH equilibrium after the initial dose of POB_1 did not alter the equilibrium response but did reduce the MCH response and the subsequent MCH-plus-NA response. This suggests that the action of the second dose of POB_2 was primarily on the muscarinic receptors rather than on the α-receptor. It is not clear if this resistance of the α-receptors to blockade is due to the presence of MCH, since partial blockade of cholinomimetic stimulation reduces the response to NA after POB blockade of α-receptors.

In some experiments, tissues were reexposed to POB_2 for greater periods to attempt to block α-receptors which were possibly missed in the original POB_1 exposure. After the original exposure, the final equilibrium to MCH-plus-NA were measured from the baseline and compared to the final equilibrium to MCH-plus-NA after the second exposure to POB_2 in the absence of any agonists (Figure 9, Table VII).

In 10 tissues tested (n=5), the addition of POB_2 for the second time drastically reduced the response to NA in the presence of MCH ($p < 0.01$) but also reduced MCH responses ($p < 0.02$). Although the second exposure of POB_2 did significantly reduce the equilibrium of MCH-plus-NA, the response to NA in the presence of MCH was still very much present when compared to MCH control ($p < 0.001$).

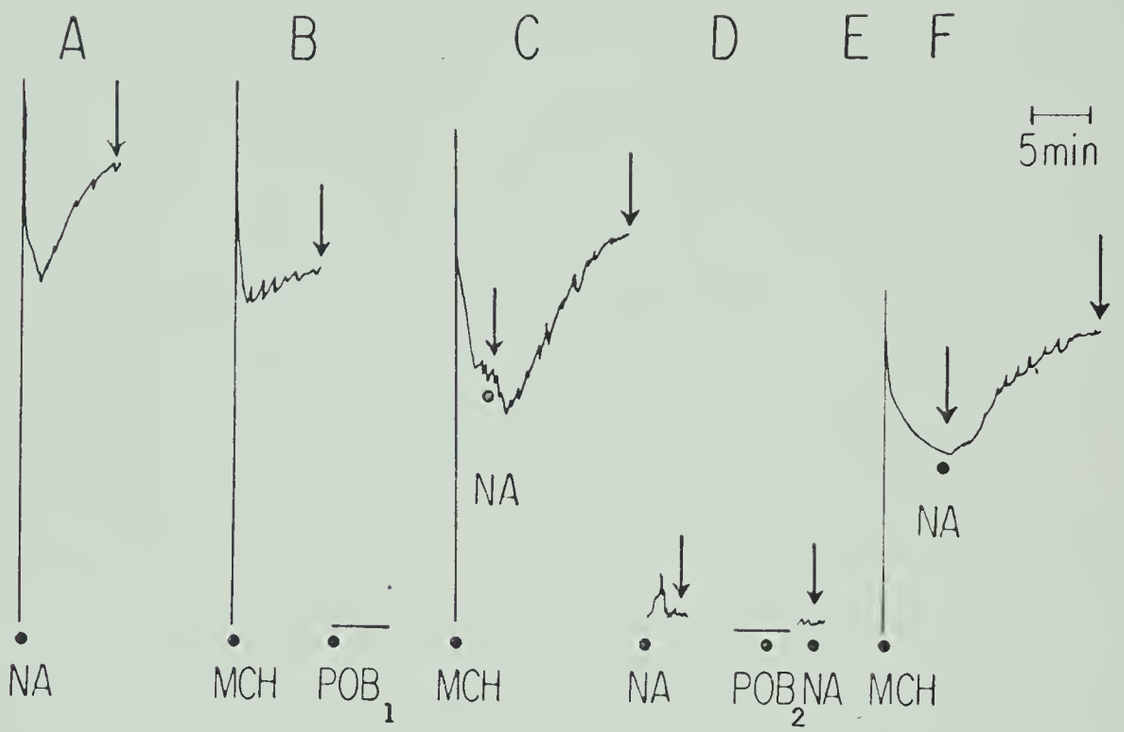

Figure 9. The effect of additional phenoxybenzamine (POB_2) on methacholine-plus-noradrenaline equilibrium in the absence of any agonists after an initial exposure to phenoxybenzamine (POB_1).

A and B represent typical responses of the stripped vas deferens to supramaximal doses (sup) of noradrenaline (14.8 - 297.0 x 10^{-5}M) and methacholine (5.1 - 15.3 x 10^{-5}M) respectively. After exposure of the tissue to POB_1 (5.9 - 8.8 x 10^{-8}M) for 5 minutes and subsequent washouts, NA (sup) in the presence of MCH (sup) elicited a response. However to eliminate α-receptors that were spared by the initial dose of POB_1 (D), additional POB_2 (2.9 - 5.9 x 10^{-5}M, for 5 minutes) were exposed to the tissue. The response to NA (sup) was essentially zero (E); however, NA (sup) was still able to elicit a response in the presence of MCH (sup)(F). Therefore, the α-receptors uncovered by MCH (sup) are apparently resistent to POB. Breaks in the diagram represent washings and vertical aroows, the measurement of equilibria responses.

TABLE VII

The effect of additional phenoxybenzamine (POB_2) in the absence of agonists after an initial treatment with phenoxybenzamine (POB_1)

Measurement of Equilibrium (mm) † from the Baseline								
Control[a]		Treatment with POB_1			Treatment with POB_2			
1 NA	2 MCH	3 (a) MCH	+ (b) NA	4 NA	5 (a) MCH	(b) +	(c) NA	6 NA
42.30	34.80	81.20[b]	43.60[c]	1.10	72.20[b]	14.70[d]	30.20[c,d]	0.31
±	±	±	±	±	±	±	±	±
5.87	4.69	4.41	6.69	0.31	5.70	2.49	2.49	0.15

† Mean ± standard error from 5 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3(a): initial contraction to MCH

3(b): equilibrium response to MCH-plus-NA

4: equilibrium response to NA

5(a): initial contraction to MCH after POB_2

5(b): equilibrium response to MCH

5(c): equilibrium response to MCH-plus-NA

[b] Significant difference ($p < 0.02$)

[c] Significant difference ($p < 0.01$)

[d] Significant difference ($p < 0.001$)

D. EFFECT OF ATROPINE (ATR) ON EQUILIBRIUM-RESPONSE

If the contraction due to NA after treatment of the tissue with POB is dependent on activation of MCH receptors, then ATR should affect the equilibrium response to NA, since normally ATR does not affect NA-induced contractions. ATR was added to tissues previously treated with POB (8.8×10^{-8}M for 5 min.) after the response to NA-plus-MCH had reached equilibrium (Figure 10). Controls designed to examine the effect of ATR on the responses to NA in normal tissues (without pretreatment with POB) were carried out.

In 4 paired control preparations, the addition of ATR ($3.6 - 14.4 \times 10^{-5}$M) did not affect the final equilibrium phase of the response to NA ($p < 0.20$, Table VIII). However, on 7 tissues pretreated with POB, the addition of ATR ($1.4 \times 10^{-7} - 7.2 \times 10^{-5}$M) significantly reduced the final equilibrium phase of the response to NA in the presence of MCH ($p < 0.001$, Table IX).

Since ATR in relatively large doses did not affect NA-induced contraction of normal guinea-pig vas deferens but did affect NA responses in the presence of MCH after treatment with POB, then it seems plausible that the effect of ATR is on or close to the methacholine receptor and not directly related to the α-receptors. Therefore, it seems reasonable that the muscarinic receptor influences the α-receptor previously blocked by POB, to allow contraction in response to NA. When the ATR is added, the MCH effect is reduced and the NA effect is similarly reduced since it is dependent on MCH

TABLE VIII

The effect of atropine on noradrenaline response

Measurement of Equilibrium (mm)† from the Baseline	
Control	Experimental
NA[a]	ATR + NA[b]
51.44 ± 4.39[c]	49.78 ± 4.49[c]

† Mean ± standard error from 5 animals

a Equilibrium response to supramaximal doses of noradrenaline (NA)

b Equilibrium response to atropine (ATR)-plus-NA

c No significant difference ($p > 0.20$)

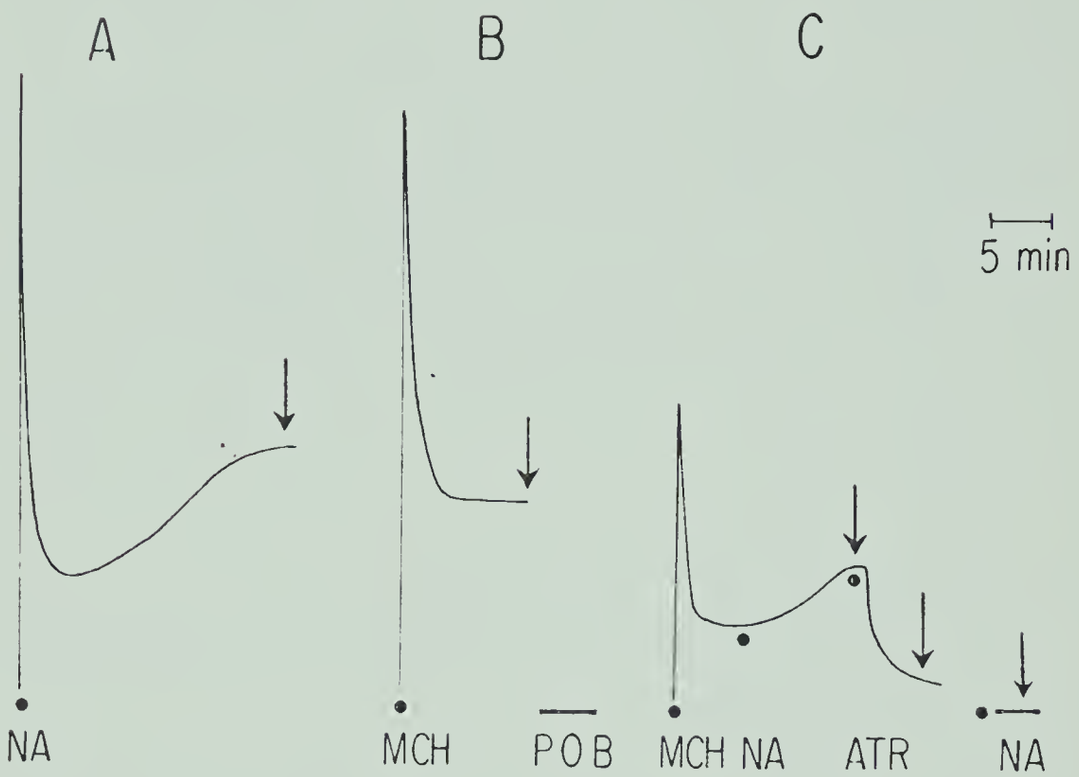

Figure 10. The effect of atropine (ATR) on noradrenaline-plus-methacholine equilibrium response after treatment of the tissue with phenoxybenzamine.

A and B represent typical responses to supramaximal doses (sup) of NA (3.0 - 23.8 x 10^{-5}M) and MCH (5.1 - 25.5 x 10^{-5}M). After exposure of the tissue to POB (8.8 x 10^{-8}M) for 5 minutes and subsequent washouts, NA (sup) in the presence of MCH (sup) elicited a response (C). However, the addition of ATR (1.4 x 10^{-7} - 7.2 x 10^{-5}M) decreased the equilibrium response (C). NA (sup) without the presence of MCH failed to contract the tissue. Therefore, the temporary reversal of POB block of α-receptors is dependent on muscarinic receptors. Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE IX

The effect of atropine (ATR) on methacholine-plus-noradrenaline equilibria response after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline			
Control[a]		After treatment with POB	
1 NA	2 MCH	3 (a) MCH + NA + (b) ATR	4 NA
55.36 ± 4.74	39.21 ± 4.81	40.85[b] ± 3.90	14.29[b] ± 2.77	0.43 ± 0.20

† Mean ± standard error from 7 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3(a): equilibrium response to MCH-plus-NA

3(b): equilibrium response to MCH-plus-NA-plus-ATR

4: equilibrium response to NA

[b] Significant difference

concentration. This was further substantiated by experiments in which the effect of MCH on α-receptor was found to be a graded response (see Section E). The exact nature of the effect of the muscarinic receptor upon the adrenoceptive α-receptor is not clear; however, it is reasonable to suppose that there may be an allosteric interaction between the muscarinic and α-receptors. This allosteric effect might be either to reversibly reduce the block by the POB (in POB remaining bound), or to increase the efficacy of α-receptors remaining free of POB or uncover "new" α-receptors. It is also conceivable that the reduction of MCH-plus-NA equilibrium by atropine may be due to a general reduction of MCH equilibrium response rather than a reduction of allosteric interaction. This in turn may also account for the reduction of the NA response. Therefore, distinction between the two possibility cannot be resolved at present.

E. DOSE-RESPONSE RELATIONSHIPS OF MCH AND ACH

Additional experiments were carried out to determine whether the effect of MCH on the α-receptors was graded or "all or none". The dose-response relationships between the concentration of MCH and the equilibrium response to the addition of NA were determined. Similarly, dose-response relationships were determined for NA response in the presence of supramaximal doses of MCH (Figure 11).

After most α-receptors had been blocked by POB (i.e., no response to supramaximal doses of NA), various doses of MCH were combined with supramaximal doses of NA; likewise doses of NA were varied and a constant (supramaximal) dose of MCH was employed.

The effect of MCH on the α-receptors was found to be a graded rather than all or none response in 3 paired preparation (Figure 11, Table X). The effects of NA (Figure 12, Table XI) with supramaximal dose of MCH, revealed that the response of NA was related to its concentration. Therefore, the effect of MCH on the α-receptor blocked by POB is not due to a "threshold phenomenon" but is dependent on the concentration of MCH, and the number of muscarinic receptors activated. This is additional proof that the decrease in equilibrium response to NA in the presence of MCH after a second exposure to POB (see Section C) was due to blocking effects of POB on the muscarinic receptors rather than on the α-receptors. The response to NA in the tissues pretreated with POB-plus-MCH was similar to that seen for NA in normal tissues.

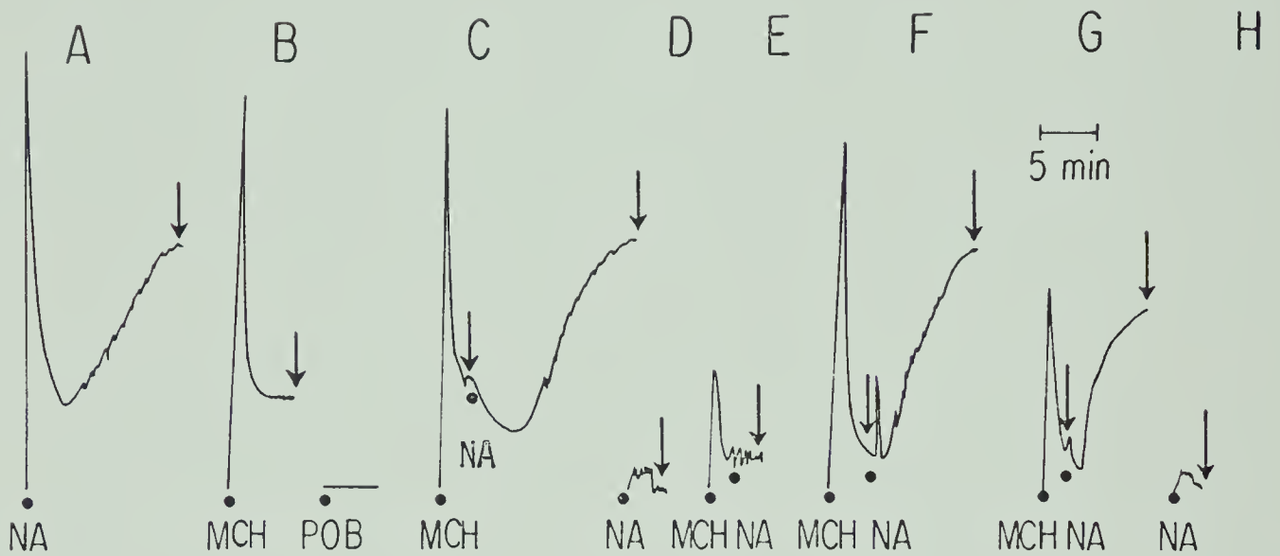

Figure 11. The dependence of noradrenaline response on the concentration of methacholine after phenoxybenzamine treatment.

A and B represent typical responses of the stripped vas deferens to supramaximal doses (sup) of NA (14.8 x 10^{-5}M) and MCH (5.1 - 25.5 x 10^{-5}M) respectively. After exposure of the tissue to POB (8.8 x 10^{-8}M) for 5 minutes and subsequent washouts, NA (sup) in the presence of MCH (sup) elicited a response (C). D and H represent NA (sup) responses in the absence of MCH. E, F and G represent responses to submaximal doses of MCH [1/500, 3/100 and 1/100 of the supramaximal doses respectively with NA (sup)]. Note that the apparent reversal of POB blockade of the α-receptors is dependent on the concentration of MCH and appear to be graded responses rather than an "all or none" effect. Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE X

The effect of varying methacholine doses with supramaximal concentration of noradrenaline after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline													
Control[a]		After treatment with POB											
1 NA	2 MCH	3 (a) MCH	3 (b) + NA	4 MCH		+ NA	5 MCH		+ NA	6 MCH		+ NA	7 NA
50	4	6	25	(1/100)	1	2	(5/100)*	2	40				0
57	28	13	23		1	2		3	38				1
48	18	17	46	(1/500)	2	2	(1/100)	9	47	(3/100)	5	33	1
40	30	25	38		1	1		4	10		2	20	1
54	26	35	55	(4/100)	1	1	(2/100)	0	1				0
56	66	35	60		17	2		18	2				0

† From 3 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) + methacholine (MCH)

Column 3(a): equilibria response to supramaximal dose of MCH

3(b): equilibria response to supramaximal dose of MCH-plus-NA

* All figures in brackets indicate ratio concentration to supramaximal doses of MCH

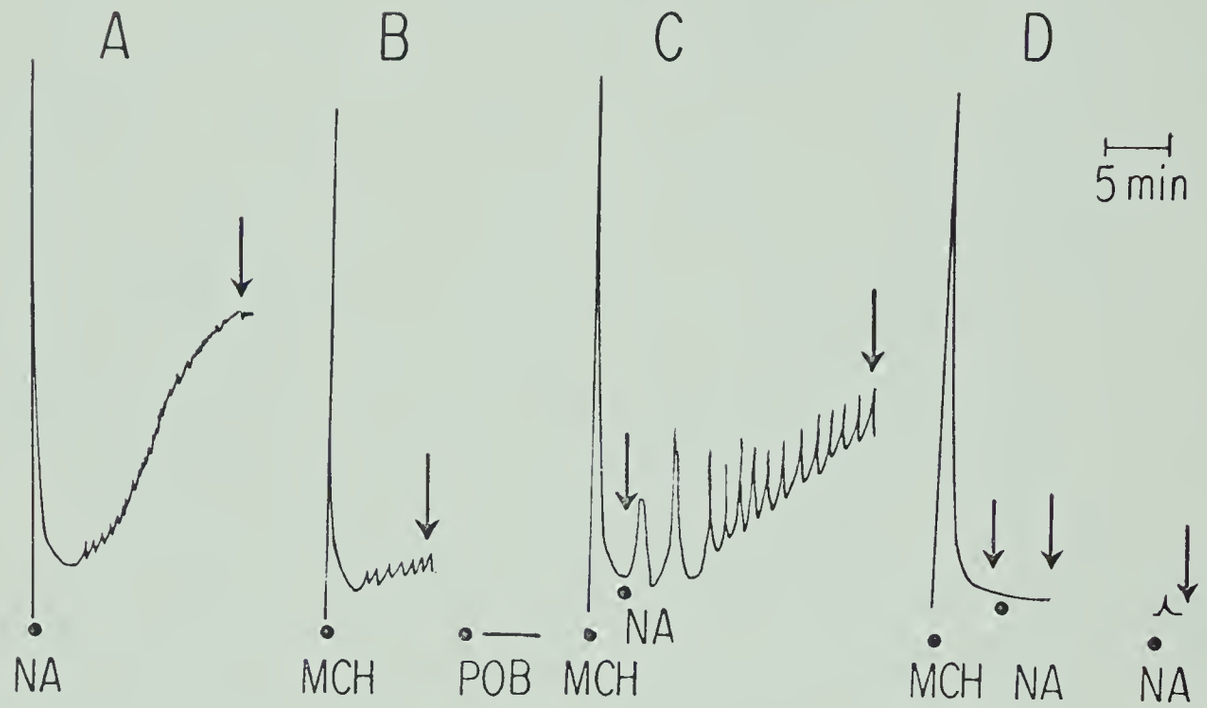

Figure 12. The addition of submaximal doses of noradrenaline to supramaximal doses of methacholine after exposure of the tissue to phenoxybenzamine.

A and B represent typical responses to supramaximal doses (sup) of noradrenaline (14.8 - 23.8 x 10^{-5}M) and methacholine (5.1 - 15.3 x 10^{-5}M) respectively. After exposure of the tissue to POB (5.9 - 8.8 x 10^{-8}M) for 5 minutes and subsequent washouts, NA (sup) in the presence of MCH (sup) elicited a response (C). However, the addition of NA (1/4 of sup) failed to elicit a response (D). Therefore the response to NA after POB treatment is related to its concentration if in the presence of MCH. Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE XI

The effect of submaximal dose of noradrenaline-plus-methacholine after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline					
Control[a]		After treatment with POB			
1 NA	2 MCH	3 (a) MCH + (b) NA		4 (a) MCH + (b) NA		5 NA
47.83	21.67	11.67[b]	13.17[b]	11.83[c]	32.67[c]	0.33
±	±	±	±	±	±	±
4.30	4.51	2.61	3.37	2.29	3.92	0.21

† Mean ± standard error from 3 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3(a): equilibrium response to MCH

3(b): equilibrium response to MCH-plus-NA (1/4 dose of control)

4(a): equilibrium response to MCH

4(b): equilibrium response to MCH-plus-NA

5: equilibrium response to NA

[b] No significant difference ($p > 0.20$)

[c] Significant difference ($p < 0.01$)

F. POSSIBILITY OF AN INDIRECT EFFECT

Burn and Rand (1965) proposed that cholinergic agonists act indirectly at sympathetic terminals by causing release of NA at the synaptic junction. Similarly, it is known that it is difficult to block NA which has been released from the nerves in the vas deferens (Burnstock and Holman, 1964; Bentley and Smith, 1967; Graham, et al., 1968).

One possibility is that MCH could release a large amount of NA by stimulating nerve endings or ganglion cells in the serous coat close to the receptors, so that exogenous NA would allow the local concentration of the agonist to exceed the threshold after the POB blockade. This requires the assumption that there is a diffusion barrier for access of exogenous NA to these receptors. It must be remembered, however, that Bentley and Smith (1967) found that POB and PHEN blocked responses to tyramine (an indirect agonist) but not to transmural stimulation.

To eliminate the possibility that MCH may be inducing an indirect effect at the synaptic junction after POB exposure, three techniques were used:

a) use of hexamethonium

b) reserpinization of the guinea-pig prior to use and subsequent exposure to POB and tyramine (which was used on occasion to determine if the initial exposure to NA must have "primed" the nerve terminals or extraneuronal uptake sites).

c) cold storage of the vas deferens

1. Hexamethonium

After exposure of the vas deferens to POB (5.9 - 8.8 x 10^{-8}M), the addition of hexamethonium chloride (3.7 - 18.2 x 10^{-5}M) to 4 paired preparations prior to MCH-plus-NA did not affect the final equilibrium response measured from the baseline ($p > 0.20$) (see Figure 13, Table XII).

The NA response in the presence of MCH after POB treatment does not appear to be a stimulating effect on ganglion cells (at least via nicotinic receptors). Moreover, removal of the serous coat of the vas deferens normally eliminates all ganglion cells which give rise to innervation of the vas deferens. Therefore, one can conclude that there are no ganglion cells within the walls of the smooth muscle itself. Chiou (1971) has suggested that hexamethonium blocks the effects of indirect agonists but not direct agonists at the postganglionic parasympathetic nerve endings in the taenia coli of the guinea-pig. If this supposition is also correct for the guinea-pig vas deferens, then the response of the α-receptor in the presence of MCH appear to be a postganglionic phenomenon. However, one could not explain that ganglion-stimulating agent, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) stimulated the stripped vas deferens (Nedergaard and Westermann, 1968). Since Nedergaard and Westermann (1968) have found that pretreatment of the vas deferens with NA potentiated the action of DMPP, it is possible that the

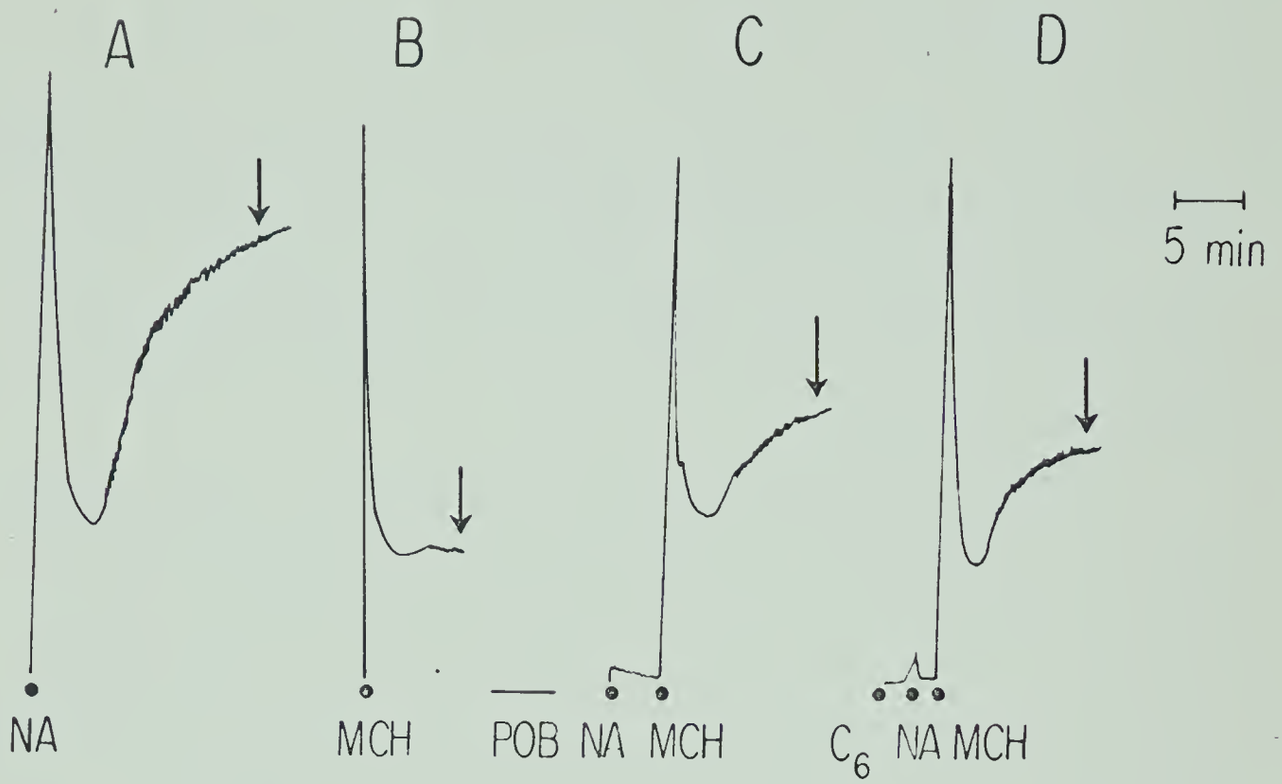

Figure 13. The effect of hexamethonium (C_6) on methacholine-plus-noradrenaline equilibrium after treatment of the tissue with phenoxybenzamine.

A and B represent typical responses of the stripped vas deferens to supramaximal doses (sup) of NA (14.8 - 17.8 x 10^{-5}M) and MCH (5.1 - 25.5 x 10^{-5}M) respectively. After exposure of the tissue to POB (5.9 - 8.8 x 10^{-8}M) for 5 minutes and subsequent washouts, NA (sup) elicited very little contraction (C). However, the addition of MCH (sup) with NA (sup) elicited a typical NA response (A). The addition of C_6 (3.7 - 18.3 x 10^{-5}M) prior to the addition of NA (sup) and MCH (sup) did not prevent the MCH from eliciting a typical NA response (D). Therefore the temporary reversal of POB blockade of α-receptors is not due to the nicotinic effect of MCH. Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE XII

The effect of hexamethonium (C_6) on noradrenaline-plus-methacholine equilibria responses after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline			
Control[a]		After treatment with POB	
[1] NA	[2] MCH	[3] NA + MCH	[4] C_6 + NA + MCH
49.12 ± 4.17	17.88 ± 3.36	30.00 ± 2.22[b]	30.12 ± 3.42[b]

† Mean ± standard error from 4 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3: equilibria responses to NA + MCH

Column 4: equilibria responses to C_6 + NA + MCH

[b] Not significant ($p > 0.20$).

effects of DMPP arise from the release of endogenous NA. Based on the observation that the effects of DMPP were blocked by cooling, hexamethonium, and triethylcholine bromide (TEC), Chiou and Long (1969) concluded that DMPP may exert an indirect effect. However, Barlow and Franks (1971) reported that DMPP had an atropine-like effect on the muscarinic receptors of the guinea-pig ileum.

2. Reserpinization

Pretreatment of the 8 guinea-pigs with reserpine (I.P., 2.3 - 2.5 mg/kg/day) for two days prior to the experiments did not eliminate the NA response in the presence of MCH after POB blockade of the α-receptors (Figure 14, Table XIII). Comparison of the final equilibrium response measured from the baseline showed that the MCH-plus-NA response was significantly greater than to MCH alone ($p < 0.001$).

Tyramine hydrochloride ($1.15 - 1.23 \times 10^{-4}$M) did not greatly affect the contraction of the tissues from reserpinized animals ($n=2$, Table XIV) in the presence of MCH after POB treatment ($p < 0.20$). Nedergaard and Westermann (1968) found that the responses to tyramine in the stripped vas deferens of guinea-pig were absent or very small. However, Nedergaard and Westermann found a marked potentiation of the tyramine response in the presence of threshold concentrations of exogenous NA.

In guinea-pig vas deferens, prior treatment with reserpine reduced the contraction produced by nerve stimulation (Hukovic, 1961; Burnstock and Holman, 1962; Bell, 1969; von Euler, 1969). However,

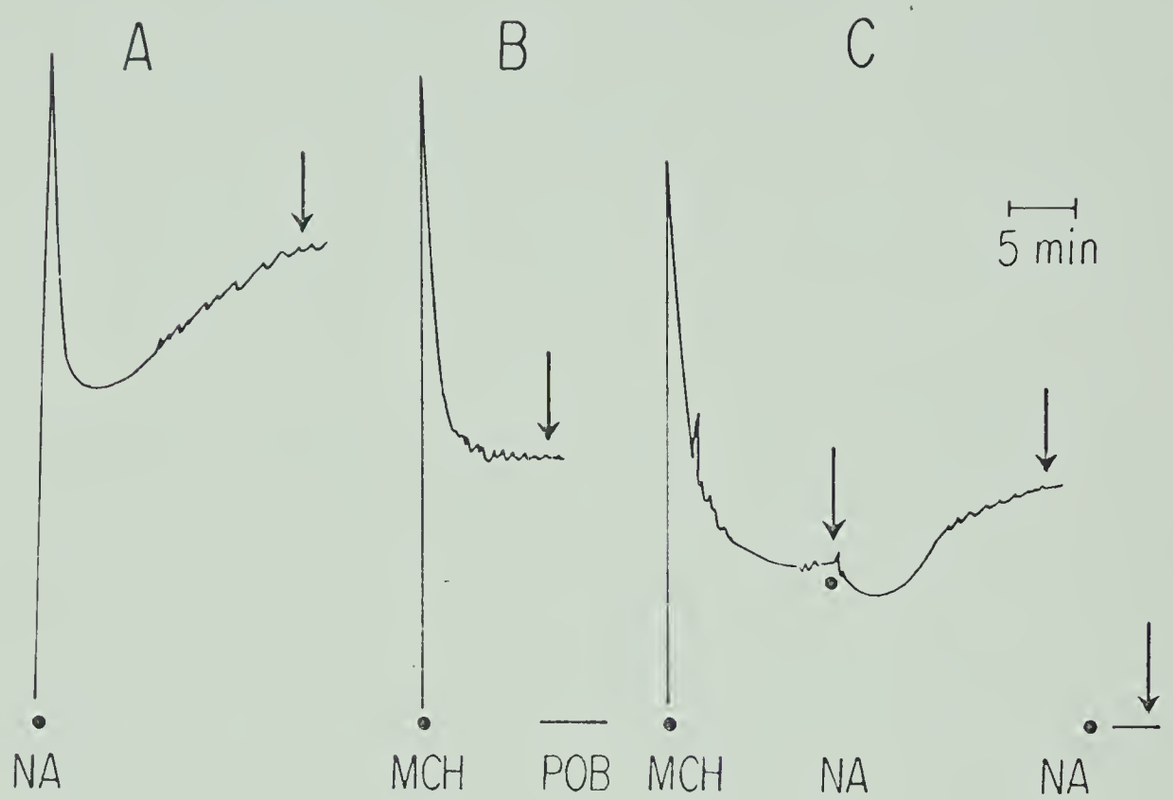

Figure 14. The effect of reserpinization of the guinea-pig prior to exposure to phenoxybenzamine on methacholine-plus-noradrenaline responses.

A and B represent typical responses of the stripped vas deferens to supramaximal doses (sup) of NA (14.8 x 10^{-5}M) and MCH (5.1 - 15.3 x 10^{-5}M). After exposure of the tissue to POB (8.8 x 10^{-8}M) for 5 minutes and subsequent washouts, NA (sup) in the presence of MCH (sup) elicited a response (C). However, NA (sup) without the presence of MCH failed to contract the tissue. Therefore, the temporary reversal of POB blockage of the α-receptors is not due to the indirect effect of MCH, i.e. release of endogenous NA by MCH. Breaks in the diagram represent washings and vertical arrows, the measurement of equilibria responses.

TABLE XIII

The effect of pretreatment of the guinea-pig with reserpine* on methacholine-plus-noradrenaline equilibria responses after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline				
Control[a]		After treatment with POB		
1 NA	2 MCH	3 (a) MCH	+ (b) NA	4 NA
51.76 ± 4.32	32.21 ± 3.47	15.21[b] ± 2.32	34.07[b] ± 5.24	0.36 ± 0.13

* 2.3-2.5 mg/kg/day for 2 days, I.P., prior to experiment

† Mean ± standard error from 7 animals

a Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3(a): equilibrium response to MCH

3(b): equilibrium response to MCH-plus-NA

4: equilibrium response to NA

b Significant difference ($p < 0.001$)

TABLE XIV

The response to tyramine in the presence of methacholine after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline			
Control[a]		After treatment with POB	
1 NA	2 MCH	3 NA	4 (a) MCH + (b) TYR
43.67 ± 13.12	20.67 ± 4.67	0.00 ± 0.00	6.33[b] ± 1.86 — 7.67[b] ± 3.18

† Mean ± standard error from 2 animals previously reserpinized for 2 days (2.3 mg/kg/day) prior to experiment

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH)

Column 3: equilibrium response to NA

Column 4(a): equilibrium response to MCH

4(b): equilibrium response to MCH-plus-TYR

[b] No significant difference ($p < 0.20$)

brief exposure to NA results in increased contraction in response to nerve stimulation (Hukovic, 1961; Birmingham and Wilson, 1963). This increase in contraction of reserpine-pretreated vas deferens which they observed after the addition of exogenous NA may be due to the existence of an extraneuronal reserpine-resistant pool which can be replenished by uptake of exogenous NA which can then be released by tyramine (Iversen, 1967). Since reserpinized tissues were exposed to exogenous NA after treatment with POB and before exposure to tyramine, it follows that if exogenous NA were replenishing an extraneuronal reserpine-resistant pool then tyramine should potentiate the response to MCH after treatment with POB. However, this is not the case. Tyramine did not potentiate the equilibrium response to MCH, but NA did potentiate this response. Therefore it follows that MCH is unlikely to act indirectly to release NA but rather seem to uncover α-receptors since tyramine, which is accepted as an indirect agonist, failed to potentiate MCH in condition where there was store of NA. It should be noted that in the rabbit arotic strips, N,N-dimethyl-2-bromophenethylamine initially blocked responses to NA and tyramine (Triggle, 1965). However, with time the response to NA was completely restored but not to tyramine even though the tissue was previously "primed" with NA.

3. Cold storage

Ambache (1946) has produced evidence which suggests that cold storage of isolated mammalian intestine leads to degeneration

of the neural elements before the response of the tissue to exogenous agonist is altered. Similarly, Kosterlitz and Lees (1964) reported that prolonged cold storage of intestinal smooth muscle leads to irreversible damage to nerve structure.

The cold storage of isolated guinea-pig ileum decreased the response to partial agonists but not to full agonists (Chiou and Long, 1969; Takegei et al., 1970). Therefore, if after cold storage of the vas deferens, the NA response in the presence of MCH is preserved despite pretreatment with POB, then the effect of MCH is not due to an indirect effect but rather to a direct effect on the post-synaptic α-receptor.

Fresh stripped vas deferens were placed in Krebs solution (100 ml) and placed in cold storage (4°C) for 2 - 7 days. After removal from cold storage, the tissues were mounted in an organ bath and allowed to equilibrate for approximately 1 hour before being tested. After cold storage for 4 days at 4°C, vas deferens from 2 animals responded to NA in the presence of MCH after prior treatment with POB. Comparison of the final equilibrium responses to MCH and MCH-plus-NA showed that the latter was significantly greater ($p < 0.01$). However, in 5 additional preparations in which the tissues were stored for 2 - 7 days (Table XV), normal response to NA (n=5) and MCH (n=3) were obtained but the experiments were not carried further because the tissues failed to return to baseline after repeated washing for more than 2 hours.

TABLE XV

The effect of cold storage on methacholine-plus-noradrenaline equilibria responses after treatment with phenoxybenzamine (POB)

Measurement of Equilibrium (mm)† from the Baseline					
Control[a]		After treatment with POB			Cold Storage (4°C)
1 NA	2 MCH	3 (a) MCH +	(b) NA	4 NA	Days
29.33 ± 3.84	13.67 ± 2.33	5.33[b] ± 1.40	14.67[b] ± 0.88	0.6 ± 0.33	4

† Mean ± standard error from 2 animals

[a] Equilibria responses to supramaximal doses of noradrenaline (NA) and methacholine (MCH).

Column 3(a): equilibrium response to MCH

3(b): equilibrium response to MCH-plus-NA

4: equilibrium response to NA

[b] Significant difference ($p < 0.01$)

If cold storage does destroy both neural elements and the response to partial or indirect agonists, then the NA response obtained in the presence of MCH after treatment with POB is due to a direct effect on the post-synaptic α-receptor. Similarly, the unusual response to NA (a rapid contraction followed by a dip below the final equilibrium response) is probably due to a post-synaptic phenomenon.

All three techniques used (utilization of hexamethonium, reserpinization, cold storage) to determine if there is any indirect effect of MCH, to cause endogenous NA release which in turn could potentiate exogenous NA, gave the same results, i.e., the effect of MCH does not depend on endogenous NA.

G. EFFECT OF 5-HYDROXYTRYPTAMINE (5-HT) ON MCH RESPONSES

Ambache et al., 1971, reported that 5-HT (1-10 µg/ml) failed to contract the stripped guinea-pig vas deferens. In our experiments in tissues without prior treatment with POB, 5-HT (12.5 - 19.8 x 10^{-5}M) also failed to contract the vas deferens. However, prior administration of 5-HT before MCH responses significantly increased the final equilibrium of MCH ($p < 0.001$) when compared with MCH alone (Table XVI). This may suggest that MCH may uncover 5-HT receptors which are not normally evident until MCH is present.

TABLE XVI

Potentiation of 5-hydroxytryptamine (5-HT) by methacholine

Measurement of equilibrium (mm)† from the baseline	
Control	Experimental
1 MCH	2 (a) 5-HT + (b) MCH
24.46[a] ± 3.37	0.92 ± 0.26
	44.00[a] ± 2.66

† Mean ± standard error from 7 animals

Column 1: equilibrium response to supramaximal dose of methacholine (MCH)

Column 2(a): equilibrium response to 5-Hydroxytryptamine (5-HT)

2(b): equilibrium response to 5-Hydroxytryptamine (5-HT) + MCH

[a] Significant difference ($p < 0.001$)

H. SUGGESTION FOR FURTHER RESEARCH

Recently Birmingham (1970) reported that denervation of the vas deferens of the guinea-pig and rat, eliminated the response to electrical stimulation and to tyramine and also caused almost complete depletion of endogenous NA as shown by absence of fluorescence. Since this method appears to destroy neural elements and yet maintains good responses to agonists (as shown by supersensitivity), it is suggested that this method would give a better evaluation of whether MCH, after treatment of the tissue with POB is indeed causing an indirect effect.

Another possibility is the use of 6-hydroxydopamine (6-OHDM) since recent work have established that 6-OHDM depletes tissues of NA (Porter et al., 1965; Lavety et al., 1965; Tranzer and Thoenen, 1968; Votavova et al., 1971, and decrease responses to sympathetic nerve stimulation (Haeusler et al., 1969).

Whether the uncovering of the α-receptor by MCH after POB is species dependent remains to be seen. However, since the response of the rat vas deferens to cholinergic drugs is relatively small compared to the responses to adrenergic drugs and also there are few β-receptors (Vohra and Reiffenstein, 1967). Therefore, the rat vas deferens could provide a better preparation for investigation of the effects of MCH on α-receptors. It is also conceivable that the partial contraction induced by MCH after treatment of the tissue with POB may have potentiated the NA response. To investigate this

possibility, the response obtained from the addition of NA, in an amount just sufficient to cause contraction of the tissue, should be compared with the response produced by the same dose of NA added after the response to MCH had reached equilibrium. If the response of NA is potentiated in the presence of MCH, then the apparent reversal of POB may be due to the partial contraction (mechanical effect) of the tissue. However, it may also be due to an allosteric effect of MCH on the α-receptor. To distinguish between the two possibilities, mechanical and allosteric, it is suggested that comparison of NA response should be made when the tissue is contracted by MCH and K^+ (see General Discussion).

Another possibility to distinguish between mechanical and allosteric effect is to record contraction of the tissue isometrically. If NA in the presence of MCH elicits a response after treatment of the tissue with POB, then MCH is truly inducing an allosteric effect on the α-receptors.

IV. GENERAL DISCUSSION

It has been shown that although nearly all alpha receptors could be blocked by POB, methacholine could permit a response to NA. How might this be occurring? It was suggested by Reiffenstein (1968) and Nakatsu and Reiffenstein (1968) that possibly conformational changes at the α-receptor may be induced by other drugs (particularly cocaine) to reverse blockade and allow α-agonists to react. This might be termed an allosteric effect, but also might result from a change in shape or "state of stretch" of the whole cell membrane produced by the contraction to MCH.

It is unlikely that MCH is causing POB to dissociate from the receptors since the same level of blockade of NA exists after washout of the MCH. Thus the POB must still remain attached to the receptors and acts in an essentially irreversible manner. It follows therefore that the permanent binding site of POB cannot be the "active" site of the receptor with which NA combines to produce its effect. This has also been suggested by Moran et al., (1970). It also follows that the induced change could result in an uncovering of the α-receptor by removal of steric hinderance of the POB attached to a nearby site rather than in a change in the reactivity of the receptor.

An interesting phenomenon was discovered by Bose (1971, unpublished material). He has shown that when POB was used to block NA contraction, the response to NA can be restored by using sodium-

free Krebs solution. However, when the normal Na^+ concentration was restored, there was no contraction to NA. Perhaps this may indicate that POB may require sodium to effect blockade and by varying the concentration of sodium it may be possible to determine the response to NA is either a graded or "all or none" response. A possible explanation for the phenomenon observed by Bose is that membrane transport mechanism for the uptake of NA requires sodium (Keen and Bogdanski, 1970). The absence of sodium may have prevented the uptake and thus allowed greater concentration of NA at the receptor sites.

Another possibility is that a decrease in sodium in the Krebs solution increases calcium influx required for the contraction mechanism, since sodium appears to compete with calcium for membrane carrier sites (Baker, et al., 1969).

Since Moran et al., (1970) have speculated that POB blocks "calcium-recognition site" rather than directly on the alpha receptor, one would be tempted to speculate that perhaps in sodium-free Krebs solution, calcium influx may be increased by utilization of carrier sites which formerly competed with sodium.

One explanation that would satisfy all these observations is that there are separate receptors for POB, NA, MCH, Histamine (HIST) and 5-HT which are all closely situated together on the membrane. POB could block any and all of these receptors either by steric hinderance, formation of a weak chemical bond between part of the POB molecule and the receptors for the agonists, or by an induced

(allosteric) change in the reactivity of the agonist receptor sites. The latter seems the most attractive hypothesis since it is known that NA, ACH, HIST and 5-HT can all specifically prevent POB attachment and binding to their respective receptors -- something that could easily be a bidirectional allosteric effect. The other possibility (steric hinderance) would not necessarily prevent POB from binding to its own receptor.

The allosteric influence of MCH could be on the NA receptors to alter its reactivity despite the continuing effect of POB, on the POB receptor to alter angle of POB attachment and thus alter steric effect, or prevent the allosteric influence of the POB receptor on the NA receptor.

In the case of the 5-HT receptor (which is not evident until MCH is present), MCH may either uncover the 5-HT receptor or cause the NA receptor to react to 5-HT. If the former is true, then this phenomenon is probably quite widespread. Philosophically, this is not surprising since the membrane surface, of which the receptor is a part, is a continuous sheet of protein; receptors of various kinds are likely to be contiguous, and alteration of one receptor by combination with an agonist is likely to affect neighbouring membrane sites particularly when one considers the nature of the conformational forces within the protein (secondary and tertiary structure).

This hypothesis can be used to explain results reported by others. For example, Nakatsu and Reiffenstein (1968) suggested that cocaine caused alteration of the reactivity of the α-receptor resulting

in increased receptor utilization. Since their experiments were done in POB-blocked tissue, it is entirely possible that they were observing a reversal of the POB blockade without dissociation of POB, by such an allosteric mechanism. However, the extent of the α-receptor blockade by POB was 40-50%. It is therefore appropriate to ask the question: what would be the extent of the potentiation of NA by cocaine if nearly all α-receptors were blocked by POB? Would the effects be similar to those of MCH on the α-receptors? If indeed cocaine is causing an allosteric effect on the α-receptors, then cocaine should, as with MCH, reverse the blocking action of POB. Perhaps cocaine requires some free α-receptors to be effective.

To distinguish between an allosteric and mechanical change at the alpha receptor, one might be able to use an agonist which reacts in a different way. After blocking α-receptors of the vas deferens of the guinea-pig with POB, NA could be added in the presence of potassium which are not blocked in doses required to block α-receptors, in the presence of potassium, but can do so with MCH, then the effect is not due to contraction-induced changes in the shape of the tissue, but due to an allosteric change at the alpha receptors. However, if reversal of α-receptor blockade occurs with potassium, then it may well be due to mechanical changes in the membrane, since potassium acts by membrane depolarization, rather than with specific receptors affected by POB.

Similarly another method to distinguish between mechanical and allosteric mechanism of MCH on α-receptor is to record the

contraction of the tissue isometrically. It is interesting to note that Rang (1971) in a review of drug-receptor interaction, has cited examples in which isometric contractions of the guinea-pig ileum and chick biventer cervicis give slopes of the Hill plot at 50% of maximum response of 1.9 and 3.3 respectively. This indicates that even under isometric conditions allosteric interactions do occur. Irrespective of the mechanism, the uncovering of the α-receptors by either of these methods may indicate that POB does not block α-receptors *per se*.

V. CONCLUSIONS

a) After treatment of the tissue with POB, NA in the presence of MCH elicited a contraction. Upon subsequent washout, the response to NA was still blocked by POB. There was no significance difference in the final equilibrium response whether NA was added prior to MCH or after the MCH response had reached equilibrium. However, there was a significant increase in the final equilibrium response of NA-plus-MCH when comparisons were made to the addition of MCH alone. The apparent reversal of POB by MCH could not be attributed to unmasking of α-receptors which had escaped blockade by POB, since the tissue responded equally to phenylephrine, a relatively pure α-agonist. Therefore, MCH may be inducing an allosteric change in the α-receptor to allow responses to NA.

b) PHEN and TOL significantly reduced the NA equilibrium response in the presence of MCH after prior treatment with POB. However, this blockade was not maintained by TOL. In control preparations, without POB pretreatment, PHEN reduced the equilibrium response to NA but TOL did not.

c) The addition of a second dose of POB during the equilibrium phase of the MCH-plus-NA response after prior treatment with POB, did not cause a decrease in the final contraction. However, upon washing the tissue, there was a significant decrease in both MCH and MCH-plus-NA equilibrium responses when the data were compared

with those obtained previously after the initial exposure of POB. This appeared to be mainly due to POB blockade of muscarinic receptors.

The second addition of POB in the absence of any agonists reduced the response to MCH alone and MCH-plus-NA. However, the response of NA in the presence of MCH was still present which may indicate the presence of α-receptors which are resistant to the action of POB.

d) In tissues without prior treatment with POB, atropine did not cause any significant decrease in NA response. However, after treatment with POB, atropine significantly decreased the MCH-plus-NA response. Since atropine did not influence the normal NA response but did affect MCH-plus-NA, then the effect of atropine is on the muscarinic receptor which in turn may be inducing an allosteric effect on the α-receptors.

e) After treatment of the tissue with POB, the effect of MCH on the α-receptors was found to be a graded rather than an "all or none" response. Therefore, the decrease in response to NA described in Section C and E may have been due to the effects of POB on muscarinic receptors rather than on the α-receptors.

f) 5-HT elicited very little or no contraction. However, in the presence of METH, it increased the final equilibrium response of MCH. This may suggest that in the presence of MCH, 5-HT receptors

were uncovered.

g) The addition of hexamethonium after exposure of the vas deferens to POB, did not affect the NA response in the presence of MCH. Pretreatment of the guinea-pig with reserpine or cold storage of the vas deferens gave similar effects. Priming of the vas deferens from guinea-pig pretreated with reserpine with NA did not elicit a response with tyramine after POB treatment. Therefore, the effects of MCH on the α-receptor after treatment of the tissue with POB is not due to an indirect effect but is probably entirely due to a post-synaptic phenomenon.

h) Results obtained suggest that muscarinic receptors may perhaps have an allosteric influence on α-receptors, either to uncover receptors sterically hindered by POB, or more probably, to alter the effects of POB on the α-receptors. However, isometric experiments and/or comparison of NA + K^+ in the presence of MCH after POB treatment should be performed to confirm the above hypothesis. A corollary is that the site of combination of POB is not the alpha receptor itself, but a site nearby.

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APPENDIX

Hill plot: log $[E/E_{max} - E]$ versus log concentration of the agonists

where: E = effect produced

E_{max} = maximal effect.

Hill plot significantly greater than one is indicative of allosteric interactions (Rang, 1971).